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Extraction of phenolic compounds with antioxidant activity from olive pomace using natural deep eutectic solvents: modelling and optimization by response surface methodology

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Abstract
This work explored the use of natural deep eutectic solvents (NADES) for extracting phenolic compounds with antioxidant activity from Uruguayan olive pomace (OP), a by-product of olive oil mills. Among nine NADES tested, lactic acid-glucose (La-Gc) was the most effective solvent for phenolic compound extraction. Further investigation focused on optimizing the extraction process using La-Gc. Response surface methodology was employed to analyze the impact of extraction temperature, water content in NADES and solid–liquid ratio on total phenols content (TPC) and antioxidant activity (FRAP and DPPH assays). Temperature and solid–liquid ratio greatly influenced TPC and antioxidant activity, while water content in NADES only showed significant influence on antioxidant activity. The optimum extraction conditions that maximized TPC and antioxidant activity were 80 °C, 68% (w/w) of water in NADES and solid–liquid ratio of 0.014 g/mL, showing a TPC of 15.56 mg GAE/g db, FRAP of 178.14 μmol FSE/g db and DPPH of 72.75 μmol TRE/g db, with hydroxytyrosol content of 1.24 mg/g db. These values were significantly higher than those of extracts obtained from conventional solvents under identical extraction conditions. These results highlight the suitability of the present extraction method using La-Gc as solvent to obtain phenolic compounds with antioxidant activity from OP.

Keywords Olive pomace · Antioxidants · Extraction · NADES · Modelling

1 Introduction

Olive pomace (OP, also called Alperujo) is the by-product from oil mills that use a two-phase extraction system to obtain extra virgin olive oil. It is a complex mixture, made up of skin, pulp, pit and water present in the olive, which gives rise to a semi-solid sludge with moisture on a wet basis between approximately 60 and 70%. In addition, it includes a mineral fraction and an organic fraction, made up of lipids, proteins, water-soluble carbohydrates, organic acids, remains of the cell wall of the olive (pectic polysaccharides, cellulose, hemicelluloses), gums, tannins and phenolic compounds [1].

After the olive oil extraction process, approximately 98–99% of the phenolic compounds present in the fruit remain in the OP, so this by-product is a rich source of this type of compounds, which have strong antioxidant activity [2–4]. Among the main phenolic compounds present in OP are hydroxytyrosol, tyrosol, oleuropein, caffeic acid, *p*-coumaric acid, verbacoside, rutin and catechol [5]. Several researchers have shown that these compounds have many benefits for human health, related to their antioxidant properties [3, 6, 7]. These compounds can be used in both the pharmaceutical and food industries. Therefore,

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the possibility of obtaining high added value compounds from a low value matrix is of great interest [8]. Additionally, the extraction of phenolic compounds can serve as a pre-treatment for the improvement of composting and bio-degradation processes of OP, since due to its phytotoxic and antimicrobial characteristics there may be problems in the development of these processes when applying them with OP directly [9–11].

The extraction of bioactive compounds from natural sources is the fundamental step to obtain natural antioxidants. The most widely used method for separating phenolic compounds from natural products is conventional solid–liquid extraction with organic solvents, such as methanol, ethanol and ethyl acetate [12, 13]. The use of these solvents has numerous disadvantages, some of them are: toxicity, large volumes required, high volatility and inflammability. These problems have led to put emphasis in the study of alternatives to the use of conventional organic solvents, following the principles of green chemistry [14, 15]. One of these alternatives is the use of green solvents called natural deep eutectic solvents (NADES). NADES are formed of natural metabolites such as sugars, alcohols, organic acids, aminoacids and amines. They are composed of a mixture of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) [16, 17]. As a result of this mixture, an eutectic liquid is obtained, with lower melting point than its individuals components [18]. These solvents have unique characteristics, such as low volatility, adjustable viscosity, non-flammability, thermal stability, adjustable solubilization power, low toxicity, and they are generally low cost and easy to prepare [19]. Most of these solvents are considered GRAS (generally recognized as safe) [20]. All the aforementioned characteristics make NADES great candidates to be used as solvents for extraction of numerous bioactive compounds from natural sources. In addition, with the natural abundance of NADES components and their inclusion in our daily dietary intake, extracts produced using NADES could be used directly in food, pharmaceutical and cosmetics formulations, reducing or eliminating the necessity for subsequent purification procedures [21]. Several investigators have successfully used NADES as extraction media to extract bioactive compounds from numerous agro-food industrial by-products [19, 21–24].

The efficiency of extraction depends on the conditions of the process. Earlier studies have highlighted the impact of certain factors (such as temperature, solid–liquid ratio, physicochemical characteristics of the solvent, among others) on the amount of phenolic compounds that can be extracted from various natural sources [25–27]. In this sense, it is crucial to optimize these parameters in order to achieve the highest possible yield of the desired compounds. To this end, Response Surface Methodology (RSM), which is a statistical experimental approach utilized for mathematical modelling [28], has become a preferred technique for standardizing process variables in numerous food processing applications and is currently being widely employed. RSM offers several advantages, such as reducing the number of required experimental measurements, providing a statistical analysis of the data, and identifying any potential interactions among variables [29, 30].

It must be taken into account that the content of phenolic compounds and the antioxidant activity of the extracts obtained from OP will not only depend on the extraction conditions and technology used, but will also depend on aspects inherent to the cultivation of the olive from which it comes (location, variety, harvest time, degree of maturity, among others) [31, 32]. In this sense, it is important to generate knowledge about the extraction of high added value compounds from OP of local origin, especially for areas where the scale of production is much smaller than that found in the main production countries of olive oil (olive oil production in Uruguay in 2019: 2500 ton/year) [33]. To the best of our knowledge, no papers have been published on the extraction of phenolic compounds with antioxidant activity through the use of NADES from OP of Uruguayan origin. In addition, some of the NADES tested in the present work have not yet been used in OP, regardless of the geographical origin of this residue.

The aim of this work was, on the one hand, to study the capacity of different novel and green NADES to extract phenolic compounds with antioxidant activity from OP produced in Uruguay. On the other hand, after the selection of the best NADES, the effect of extraction conditions (temperature, water content in NADES and solid–liquid ratio) on the total phenols content and antioxidant activity were studied and subsequently optimized, with quantification of hydroxytyrosol content and further comparison to extracts obtained with conventional solvents.

2 Materials and methods

2.1 Raw materials

OP composed of 50% mixture of Arbequina and Picual varieties was obtained from a local olive oil mill (Canelones, Uruguay) using a two-phase extraction system, with initial moisture of 72.1 ± 0.6% (wet basis). It was dried in a pilot tray drier for 48 h at 35 °C (final moisture: 4.4 ± 0.1%, wet basis). In both cases the moisture content was determined in an oven at 105 °C until constant weight [34]. Dried OP was grinded with a cutting mill (IKA MF 10, Staufen, Germany).

The particle size distribution of dried and milled OP (determined through sieve assay) was: 44.7% between 1.41 and 1.00 mm, 22.3% between 1.00 and 0.84 mm, 13.8% between 0.84 and 0.60 mm and 18.9% between 0.60 and 0.42 mm. Mean particle diameter was estimated in 0.94 mm by weighting of the different fractions obtained in the sieve assay. Initial total phenols content of dried OP was 14.48 ± 0.55 mg GAE/g db, determined by exhaustive multistage extraction experiments according to the procedure described by Xavier et al. [35], using 80% (v/v) methanol as extraction solvent at 50 °C, with a solid–liquid ratio of 1.00 g of dried OP per 20 mL of solvent and 60 min of extraction in each stage, and analyzed by the method described in Sect. 2.5. The dry sample was kept at – 18 °C until later use.

2.2 Chemicals and reagents

Choline chloride (>98%), choline bitartrate (>97%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ >99%), (±)-6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxilic acid (trolox >98%), 2-(3,4-dihydroxyphenyl) ethanol (hydroxytyrosol, analytical standard), glucose anhydrous (>99%), propylene glycol (>99%), glycerol (>99%) and lactic acid (85%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Folin-Ciocalteu's reagent, sodium carbonate anhydrous, acetonitrile (HPLC grade) and gallic acid (99%) were purchased from Merck (Darmstadt, Germany). Ferric chloride hexahydrate was purchased from Fluka Analytical (St. Louis, MO, USA). Sodium acetate anhydrous, trifluoroacetic acid and glacial acetic acid were purchased from Carlo Erba Reagents (Val de Reuil, France). Ferrous sulfate heptahydrate was purchased from Cicarelli Reagents (Santa Fe, Argentina).

2.3 Preparation of NADES

Nine different NADES were prepared according to the method described by Dai et al. [36], based on heating at 80 °C and stirring until a homogeneous liquid formed. Three types of HBA were used: choline chloride, choline bitartrate and lactic acid; in combination with different types of HBD (lactic acid, citric acid, glycerol, propylene glycol and glucose). In all cases, distilled water was added in a relation of 25% (w/w). The composition of each NADES is shown in Table 1, where the molar ratio of each NADES was selected based on bibliography [37, 38]. Viscosity was measured with a Brookfield DV2T viscometer (Brookfield Engineering Laboratories, Inc., MA, USA) with and enhanced UL adapter coupled to a circulating water bath (WiseCircu WCR-P12, Daihan, Korea), at 60 °C and 5–30 rpm range. pH was measured with a Hanna Instruments model HI 2210 pH meter (Hanna Instruments, RI, USA).

2.4 NADES screening

A NADES screening was carried out to study the effect of the solvent on the phenolic content and the antioxidant activity of OP extracts. Extraction experiments were performed in 60 mL dark glass flasks inside a thermostatic shaking water bath (Biobase SWB-A, Shandong, China). All extractions were carried out at constant temperature (60 °C) for 80 min at 210 rpm. Solid–liquid ratio of 1.60 g of dried OP per 20 mL of NADES was used. Then, extracts were centrifuged at 6000 rpm (Hermle Z 206A, Wehingen, Germany) for 15 min, the supernatant was recovered and stored at -18 °C until further analysis. Extractions for each of the nine NADES were performed in triplicate. The results obtained at this stage have been the basis for selecting the solvent in a second series of experiments planned according to Sect. 2.6.

Table 1 NADES used in extraction experiments

Components	Abbreviation	Molar ratio
Choline bitartrate: Citric acid	BtC-Ct	1:1
Choline bitartrate: Glucose	BtC-Gc	1:1
Choline bitartrate: Lactic acid	BtC-La	1:1
Choline chloride: Citric acid	ChC-Ct	1:2
Choline chloride: Glucose	ChC-Gc	2:1
Choline chloride: Glycerol	ChC-Gy	1:2
Choline chloride: Lactic acid	ChC-La	1:2
Choline chloride: Propylene glycol	ChC-Pg	1:1
Lactic acid: Glucose	La-Gc	5:1

2.5 Total phenols content and antioxidant activity

Total phenols content (TPC) was determined by the Folin-Ciocalteu method [39], according to the procedure described by Xavier et al. [40]. The results were expressed as mg of gallic acid equivalents per g of OP in dry basis (mg GAE/g db).

Antioxidant activity was measured with two methods: Ferric reducing antioxidant power assay (FRAP) and free radical scavenging capacity assay using 2,2-diphenyl-1-picrylhydrazyl reagent (DPPH). FRAP was implemented following the procedure described by Piwowarska and González-Alvarez [41]. A calibration curve made of standard solutions of $FeSO_4 \cdot 7\ H_2O$ (100–1000 µmolL^{-1}) was used. Results were expressed as μmol of ferrous sulfate equivalents per g of OP in dry basis (μmol FSE/g db). DPPH was implemented as described by Piwowarska and González-Alvarez [41] with minor modifications. Briefly, an aliquot of 0.3 mL of previously diluted extract was added to 2.7 mL of freshly prepared DPPH radical solution (6.0e−5 molL^{-1} in 80% (v/v) methanol). After 30 min remaining in darkness at ambient temperature, the absorbance was measured at 517 nm. The % of inhibition (PI) was calculated according to:

$$\mathrm{PI} = \frac{100(A_o - A_s)}{A_o} \tag{1}$$

where A_s is the absorbance of the extract solution and A_o is the absorbance of the control sample prepared without extracts. A calibration curve made of standard solutions of trolox (0.04−0.75 mmolL^{-1}) in 80% (v/v) methanol was used. Results were expressed as μmol of trolox equivalents per g of OP in dry basis (μmol TRE/g db).

2.6 Experimental design

For the selected solvent from the NADES screening, an extraction process modelling was performed. A RSM approach with a three factor and three level Box-Behnken design was used. The influence of the independent variables temperature (X_1, with levels 40, 60 and 80 °C), water content in NADES (X_2, with levels 20, 45 and 70% (w/w)) and solid–liquid ratio (X_3, with levels 0.01, 0.08 and 0.15 g of OP in db per mL of NADES) in the dependent variables TPC and antioxidant activity (FRAP and DPPH) was investigated. The different levels of the independent variables along with the extraction time were selected based on results from preliminary experimentation (data not shown). Twelve experiments with three center point replicas were performed (in random order), and the responses were fitted with a second-order polynomial model:

$$Y = \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=2}^{3} \beta_{ij} X_i X_j \tag{2}$$

where β_o is the constant coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient, all of them relative to the dependent variable Y. X_i and X_j are the levels of the independent variables coded according to the following equation:

$$X_i = \frac{x_i - x_{Mi}}{\Delta x_i} \tag{3}$$

where x_i and X_i represents the real and coded value of the independent variable i, respectively; x_{Mi} represents the value of the independent variable i at the central point and Δx_i refers to the variation interval of the independent variable i.

Comparative extraction experiments with conventional solvents were also carried out. Water and 70% (v/v) ethanol were used under the NADES optimum extraction conditions determined with the Box-Behnken design. All extractions and post separation were executed with the experimental set-up described in Sect. 2.4.

2.7 Optimization methodology

In order to optimize the main parameters involved in the extraction process, the desirability function methodology described by Derringer and Suich [42] was used. The main concept of this approach involves calculating a desirability value for each of the k responses. This value serves as an indicator of how closely the fitted value aligns with the desired value when using the optimal factor settings. These individual desirability values are then combined to create an overall desirability for a set of k response variables. The desirability function (both individual and overall) operates on a scale ranging from zero, representing a completely undesirable response, to one, indicating a fully desired response.

In the present work, the optimization objective corresponds to the simultaneous maximization of the responses TPC and antioxidant activity measured by FRAP and DPPH, with respect to the independent variables temperature, water content in NADES and solid–liquid ratio. For each of the three responses, the minimum and maximum acceptable values corresponds to the minimum and maximum experimental values obtained from the extraction experiments. Predicted values obtained from the model of the response k (y_k) are converted to individual desirability values (d_k) with the following equation:

$$d_k = \begin{cases} 0 & \text{if} \quad y_k \le y_k^{min} \\ \left(\dfrac{y_k - y_k^{min}}{y_k^{max} - y_k^{min}} \right) & \text{if} \quad y_k^{min} < y_k < y_k^{max} \\ 1 & \text{if} \quad y_k \ge y_k^{max} \end{cases} \tag{4}$$

where y_k^{min} and y_k^{max} corresponds to the minimum and maximum acceptable values of the response k, respectively. The overall desirability function (D) is defined as the geometric mean of the three individual desirability functions (d_1, d_2 and d_3), given by:

$$D = \sqrt[3]{d_1 d_2 d_3} \tag{5}$$

2.8 HPLC-DAD analysis

HPLC analysis was performed on a Shimadzu chromatography system equipped with diode array detector (SPD-M10A). A C18 column (250 x 4.6 mm, 5 µm) was used for separation (RP-18 endcapped Purospher STAR Hibar RT, Germany). Hydroxytyrosol content analysis was performed as described by Rubio-Senent et al. [2] with minor modifications. The mobile phases were 0.01% trifluoroacetic acid in MilliQ water (solvent A) and acetonitrile (solvent B) with the following gradient elution: 0–30 min, 95–75% A; 30–45 min, 75–50% A; 45–47 min, 50–0% A; 47–50 min, 0–75% A; 50–52 min, 75–95% A, then analysis continues until 55 min. The injection volume was 20 µL, flow rate was 1.0 $mLmin^{-1}$ and oven temperature was 25 °C. Hydroxytyrosol content was quantified based on integration of peaks at 280 nm using external standard calibration curve (5–40 mgL^{-1}).

2.9 Statistical analysis

ANOVA test, Tukey test, Pearson correlation coefficient and response surface analysis were carried out with software Infostat version 2018e [43], in all cases with a 95% confidence level. Contour plots were performed with package PGFPlots of the LaTeX document preparation system. Desirability function and optimization were carried out with Matlab R2015a software.

3 Results and discussion

3.1 Screening of NADES

Nine different NADES were investigated for its use as solvent for the extraction of phenolic compounds from OP. In order to characterize the physicochemical behavior of the different NADES, viscosity and pH were measured and the results are presented in Table 2, along with the properties of reference solvents water and 70% (v/v) ethanol. Water was added to all NADES in a proportion of 25% (w/w) in order to reduce the viscosity and improve mass transfer between liquid and solid phases [44]. However, in all cases NADES presented higher viscosity than water and 70% (v/v) ethanol. Adding a higher percentage of water further reduces the viscosity, but the hydrogen bonding structure characteristic of NADES could be broken, obtaining a simple solution of its components [17]. pH values of NADES ranged between 0.35 and 4.29. The lower values correspond to NADES formed with choline chloride as HBA and an organic acid as HBD (citric acid or lactic acid), and in the case of lactic acid as HBA and glucose as HBD.

Table 2 Physicochemical characteristics of solvents used in extraction experiments

NADES	Viscosity (cP)	pH
BtC-Ct	38.04 ± 1.20	2.63 ± 0.01
BtC-Gc	34.56 ± 1.20	3.99 ± 0.01
BtC-La	19.80 ± 1.20	3.81 ± 0.01
ChC-Ct	22.08 ± 1.20	0.35 ± 0.01
ChC-Gc	11.48 ± 0.20	4.29 ± 0.01
ChC-Gy	5.14 ± 0.20	3.36 ± 0.01
ChC-La	5.52 ± 0.20	0.99 ± 0.01
ChC-Pg	5.44 ± 0.20	3.23 ± 0.01
La-Gc	8.08 ± 0.20	0.61 ± 0.01
70% (v/v) ethanol	1.63 ± 0.10	6.53 ± 0.01
Water	0.73 ± 0.10	6.83 ± 0.01

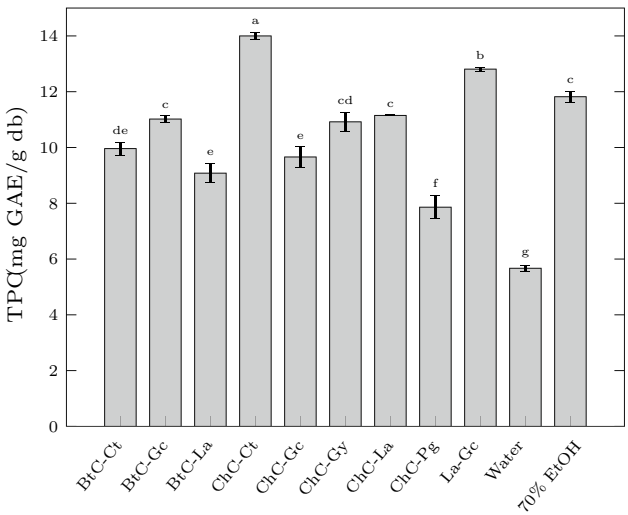

Fig. 1 Total phenols content (TPC, mg GAE/g db) of extracts from OP using NADES, water or 70% (v/v) ethanol as solvents. Mean values ± standard deviation of three replicates. Values with different letters are significantly different (Tukey test, $p < 0.05$)

Figure 1 shows the TPC of extracts from OP using NADES, water or 70% (v/v) ethanol as solvents. It is observed that in all cases, NADES extracts presented statistically higher TPC than water extracts (5.67 mg GAE/g db). TPC of ChC-Ct and La-Gc extracts (14.00 mg GAE/g db and 12.81 mg GAE/g db, respectively) were significantly higher than 70% (v/v) ethanol extracts, while no significative differences where found between TPC of 70% (v/v) ethanol, BtC-Gc, ChC-La and ChC-Gy extracts (11.82 mg GAE/g db, 11.02 mg GAE/g db, 11.15 mg GAE/g db and 10.92 mg GAE/g db, respectively). The highest TPC correspond to extracts obtained from ChC-Ct and La-Gc, both with the lowest pH, as can be seen in Table 2. Interestingly, the highest TPC corresponds to the use of ChC-Ct as solvent, which presented one of the highest viscosity among all the NADES tested. In this particular case, the pH of the solvent seems to show a greater influence than the viscosity of the solvent on the TPC of the extracts obtained. Several investigators have reported the importance of low pH values of NADES on the extraction of phenolic compounds from different vegetable matrices [19, 45, 46]. In particular, according to what was reported by Fan et al. [47] and Ying et al. [48], the improvement in the extraction of phenolic compounds through the use of ionic liquids (whose structure and interactions with phenolic compounds is similar to NADES) with low pH may be due to an increase in hydrogen bond interactions between the solvent and the phenolic compounds, especially in cases where the pH is lower than the pK_a of the phenols, where the molecular form predominates.

In relation to the influence of the HBA used to prepare the NADES (with the same HBD) on the TPC of the different extracts, from Fig. 1 it can be observed lower TPC when using choline bitartrate instead of choline chloride, except for the extract obtained with BtC-Gc. This exception is remarkable, since BtC-Gc presented higher viscosity and lower pH than ChC-Gc. As previously commented, this is another case where the pH of the solvent seems to show a greater influence than the viscosity of the solvent on the TPC of the extracts obtained.

Figure 2 shows the antioxidant activity measured with FRAP and DPPH of extracts from OP using NADES, water or 70% (v/v) ethanol as solvents. In general, it is observed that NADES extracts presented statistically higher FRAP and DPPH values than the corresponding values of water extracts. The exceptions correspond to extracts from BtC-Ct and ChC-Ct in the case of FRAP and to extracts from BtC-Ct and BtC-La in the case of DPPH. In both cases, no significant differences were found with water extracts. The highest FRAP values correspond to extracts obtained from ChC-La, BtC-La, La-Gc, BtC-Gc and 70% (v/v) ethanol (122.76 μmol FSE/g db, 119.51 μmol FSE/g db, 116.81 μmol FSE/g db, 112.83 μmol FSE/g db and 114.82 μmol FSE/g db, respectively; no significant differences were found between them). The highest DPPH values correspond to extracts obtained from La-Gc, ChC-La and 70% (v/v) ethanol (61.64 μmol TRE/g db, 59.57 μmol TRE/g db and 58.75 μmol TRE/g db, respectively; no significant differences were found between them). It should be noted that the extracts obtained from ChC-Ct had lower values of antioxidant activity compared to the other extracts, despite having the highest value of TPC. This highlights the importance of considering both analyzes of phenolic compounds and antioxidant activity to evaluate the capacity of a solvent to extract these compounds, since each method is based on different interactions between the phenolic compounds and the reagents involved, with different mechanisms of action [49]. By using more than one method simultaneously, it is possible to obtain complementary information.

In summary, the selection criteria of the best solvent based on the highest TPC and antioxidant activity values indicates that solvent La-Gc is the best and will be used in the following extraction experiments. Good results in the use of La-Gc as a solvent in the extraction of phenolic compounds with antioxidant activity from various types of agro-industrial waste compared to the use of other NADES were reported by several researchers [50–52], in particular for the case of OP [19, 53].

3.2 Modelling of the extraction process by RSM

RSM was used to study the effect of extraction conditions (temperature, water content in NADES and solid–liquid ratio) on the response variables TPC and antioxidant activity (FRAP and DPPH), using La-Gc as solvent. The experimental conditions and results of the experimental design are shown in Table 3. TPC varied from 9.89 to 15.68 mg GAE/g db, while antioxidant activity measured with FRAP varied from 94.34 μmol FSE/g db to 171.07 μmol FSE/g db and antioxidant activity measured with DPPH varied from 22.52 μmol TRE/g db to 70.05 μmol TRE/g db.

Table 4 shows ANOVA results for the three response variables. On the one hand, the three models (one for each of the response variables) showed p-values lower than 0.0001, which means that the models are highly significant. On the other hand, the insignificant lack of fit values ($p > 0.05$) indicates that each of the models are adequate to predict the corresponding response. Table 5 shows the regression coefficients (in coded variables) and the coefficients of determination for the TPC, FRAP and DPPH models after backwards elimination of the insignificant terms ($p > 0.05$). The three models presented high values of coefficient of determination (R^2) and adjusted coefficient of determination (R^2_{adj}). The values of the predicted coefficient of determination (R^2_{pred}) were in good agreement with R^2_{adj}. The aforementioned indicates a high degree of correlation between experimental and predicted values for the three models.

As can be seen in Table 5, TPC was highly influenced by extraction temperature (linear coefficient β_1, $p < 0.001$ and quadratic coefficient β_{11}, $p < 0.05$) and solid–liquid ratio (linear coefficient β_3, $p < 0.001$ and quadratic coefficient β_{33},

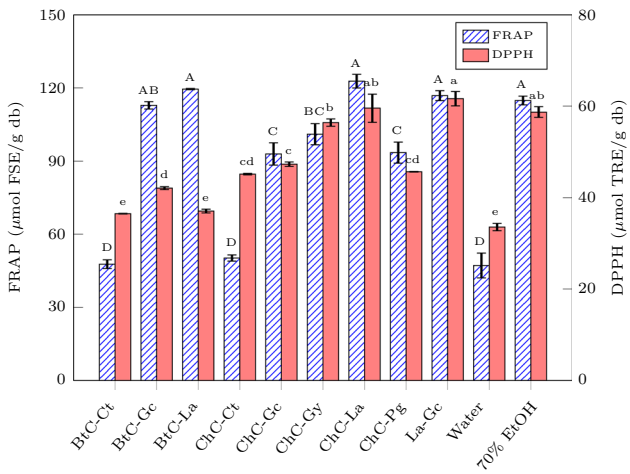

Fig. 2 Antioxidant activity measured with FRAP (μmol FSE/g db) and DPPH (μmol TRE/g db) of extracts from OP using NADES, water or 70% (v/v) ethanol as solvents. Mean values ± standard deviation of three replicates. Values with different letters are significantly different (Tukey test, $p < 0.05$). Upper-case letters correspond to FRAP values, lower-case letters correspond to DPPH values

Table 3 Experimental conditions and results for the extraction of phenolic compounds from OP according to a Box-Behnken design

Exp	Variables[a]			Responses[b]		
	X_1	X_2	X_3	TPC	FRAP	DPPH
1	40 (– 1)	20 (– 1)	0.08 (0)	10.86	94.34	27.78
2	80 (1)	20 (– 1)	0.08 (0)	15.68	136.86	33.72
3	40 (– 1)	70 (1)	0.08 (0)	10.66	98.85	26.63
4	80 (1)	70 (1)	0.08 (0)	14.35	137.75	33.88
5	40 (-1)	45 (0)	0.01 (– 1)	11.24	143.53	54.63
6	80 (1)	45 (0)	0.01 (– 1)	14.93	171.07	70.05
7	40 (– 1)	45 (0)	0.15 (1)	9.89	111.56	33.11
8	80 (1)	45 (0)	0.15 (1)	13.67	151.95	40.30
9	60 (0)	20 (-1)	0.01 (– 1)	12.89	132.33	50.75
10	60 (0)	70 (1)	0.01 (– 1)	12.59	168.72	65.44
11	60 (0)	20 (-1)	0.15 (1)	10.19	116.71	35.08
12	60 (0)	70 (1)	0.15 (1)	10.72	113.57	22.52
13	60 (0)	45 (0)	0.08 (0)	12.63	131.73	33.24
14	60 (0)	45 (0)	0.08 (0)	12.10	122.42	30.46
15	60 (0)	45 (0)	0.08 (0)	12.70	130.08	30.72

[a] X_1: temperature (°C); X_2: water content in NADES (% w/w); X_3: solid–liquid ratio (g/mL). Real and (coded) values

[b] TPC: total phenols content (mg GAE/g db); FRAP: antioxidant activity (μmol FSE/g db); DPPH: antioxidant activity (μmol TRE/g db)

$p < 0.05$), with statistically insignificant influence of water content in NADES and interactions of the independent variables ($p > 0.05$). Antioxidant activity measured with FRAP was influenced by the extraction temperature (linear coefficient β_1, $p < 0.001$), water content in NADES (linear coefficient β_2, $p < 0.05$ and quadratic coefficient β_{22}, $p < 0.01$) and solid–liquid ratio (linear coefficient β_3, $p < 0.001$and quadratic coefficient β_{33}, $p < 0.001$), with statistically significant contribution of the interaction between water content in NADES and solid–liquid ratio (β_{23}, $p < 0.01$). In the case of antioxidant activity measured with DPPH, most of the terms proposed in the model were statistically significant (linear and quadratic coefficients for extraction temperature: β_1 with $p < 0.001$and β_{11} with $p < 0.001$, respectively; quadratic coefficient for water content in NADES: β_{22} with $p < 0.01$; linear and quadratic coefficients for solid–liquid ratio: β_3 and β_{33} respectively, both with $p < 0.001$; interaction between extraction temperature and solid–liquid ratio: β_{13} with $p < 0.05$ and interaction between water content in NADES and solid–liquid ratio: β_{23} with $p < 0.001$).

Table 4 ANOVA for the reduced response surface models

Response[a]	Source	Sum of square	Df	Mean square	F-value	p-value
TPC	Model	41.69	4	10.42	49.90	<0.0001
	Residual	2.09	10	0.21		
	Lack of fit	1.87	8	0.23	2.18	0.3528
	Pure error	0.22	2	0.11		
FRAP	Model	6781.32	6	1130.22	34.47	<0.0001
	Residual	262.33	8	32.79		
	Lack of fit	212.97	6	35.49	1.44	0.4649
	Pure error	49.36	2	24.68		
DPPH	Model	2851.50	7	407.36	117.28	<0.0001
	Residual	24.31	7	3.47		
	Lack of fit	19.60	5	3.92	1.66	0.4167
	Pure error	4.72	2	2.36		

[a] TPC: total phenols content (mg GAE/g db); FRAP: antioxidant activity (μmol FSE/g db); DPPH: antioxidant activity (μmol TRE/g db)

Table 5 Regression coefficients and coefficients of determination for the reduced response surface models (in coded variables)

	TPC	FRAP	DPPH
β_o	12.346	128.252	31.473
β_1	1.998 ***	18.669 ***	4.475 ***
β_2	–	4.831 *	—
β_3	−0.898 ***	−15.233 ***	−13.733 ***
β_{12}	–	–	—
β_{13}	–	–	−2.058 *
β_{23}	–	−9.883 **	−6.813 ***
β_{11}	0.639 *	—	2.552 *
β_{22}	–	−11.433 **	−3.523 **
β_{33}	−0.651 *	16.145 ***	15.497 ***
R^2	0.952	0.963	0.992
R^2_{adj}	0.933	0.935	0.983
R^2_{pred}	0.892	0.815	0.930

TPC: total phenols content (mg GAE/g db); FRAP: antioxidant activity (μmol FSE/g db); DPPH: antioxidant activity (μmol TRE/g db). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

For a better visualization of the effects of extraction conditions on TPC and antioxidant activity, contour plots are shown in Figs. 3, 4 and 5. It should be noted that there is only one contour plot for TPC, since as previously mentioned, the effect of water content in NADES on TPC was statistically not significant.

In the case of extraction temperature, it is clear from the contour plots that an increase in this variable generates increases in TPC and antioxidant activity. Higher temperatures lead to an improvement in the mass transfer of phenolic compounds from the solid matrix to the solvent, mainly due to an increase in the diffusivity of these compounds and a decrease in the viscosity and surface tension of NADES [27, 54]. However, on the one hand, it must be taken into account that high temperatures can lead to the decomposition of phenolic compounds, mainly flavonoids [27, 51, 55]. On the other hand, NADES can suffer degradation at high extraction temperatures, although in general it has been reported that the degradation of NADES occurs at temperatures higher than those used in this work [56, 57]. In the present work, no apparent degradation of NADES was observed (evaluated as absence of color changes, absence of phase separation and absence of precipitation of components when subjecting NADES to the temperatures and extraction times tested). With respect to phenolic compounds, although the degradation of individual compounds was not evaluated by HPLC, it has been reported that the main phenolic compounds present in OP do not suffer degradation when extracted with temperatures higher than those used in the present work [2, 58]. This agrees with the trend obtained in the TPC values in the temperature range tested in this work.

Regarding the solid–liquid ratio, an increase in TPC and antioxidant activity is observed as the amount of OP added to NADES decreases. In the particular case of TPC, an increase in this variable is observed with the decrease in the solid–liquid ratio up to values close to 0.04 g db/mL, from which the variation in TPC becomes relatively low. This agrees with the fundamental principles of mass transfer, where the concentration gradient between the solid and the liquid bulk serves as the driving force. This gradient is more pronounced when a lower solid–liquid ratio is employed, improving the transfer of phenolic compounds with antioxidant activity from the solid to the solvent [25]. However, greater quantities of solvent typically have a limited impact on the quantity of phenolic compounds that can be extracted, which is undesirable from both cost and environmental perspectives [26].

In relation to the water content in NADES, an increase in antioxidant activity values is observed with increasing water content, with no significant effect of this variable on the TPC values. The increase in antioxidant activity occurs up to values close to 55% (w/w) of water content, from which the increase is relatively low. A slight decrease in the values of antioxidant activity measured with FRAP is observed for values of water content above 60% (w/w). As commented in Sect. 3.1, by increasing the proportion of water in NADES, the viscosity could be further reduced, but this may disrupt the distinctive hydrogen bonding structure inherent to NADES, resulting in a solution comprised of its individual components. Therefore, the addition of water to NADES could lead to an improvement in mass transfer up to a certain point, after which there could be a decrease in the transfer of phenolic compounds with antioxidant activity from the solid matrix to the solvent [17]. Several researchers have reported this effect in the extraction of phenolic compounds with antioxidant activity from agro-industrial residues using NADES as solvent [27, 50, 55, 59]. Interestingly, in general the reported values

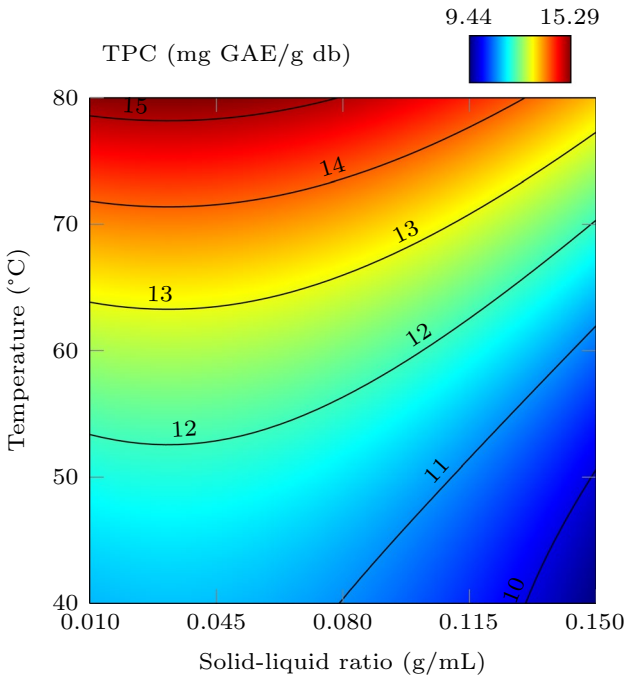

Fig. 3 Contour plot representing the TPC of extracts from OP using La-Gc as extraction solvent, as a function of solid–liquid ratio and temperature

of water content in NADES from which the transfer of these compounds from the solid matrix to the liquid begins to decrease are lower (in the order of 20–40% (w/w)) than what was found in this work. In some of these cases, the decrease in the amount of bioactive compounds extracted as the percentage of water in NADES increases is justified by a disruption in the hydrogen bonding structure, reducing the interactions between the solvent and the target molecules. In other cases, such decrease is justified by a change in the polarity of the solvent, which, depending on the type of molecules to be extracted (with low solubility in water), impairs the interaction between the solvent and the bioactive compounds to be extracted. Pisano et al. [60] studied the effect of dilution with water on the structure of the NADES La-Gc in 5:1 molar ratio, among others. They concluded that in the case of this NADES, the supramolecular structure of hydrogen bonds was maintained even with water contents of up to 80% (w/w). With the addition of the flavonoid quercetin, they found that the hydrogen bonding structure was maintained up to levels of added water of around 50% (w/w). These phenomena could explain what was found in the present work, where it is possible to obtain good results in terms of the amount of phenolic compounds with antioxidant activity extracted from OP by using La-Gc with a relatively high water content, which is desirable from an economic point of view.

As a final comment, although there is a certain relation between TPC and antioxidant activity, in the present work a linear correlation between TPC and antioxidant activity determined by FRAP and DPPH was not found (Pearson correlation coefficients of 0.680 and 0.379, respectively). However, a good linear correlation was found between both methods for determining antioxidant activity (Pearson correlation coefficient of 0.835). These results are in line with the reported by other investigators [31, 50, 61]. In general, the antioxidant activity of phenolic compounds depends on each individual molecule, as well as on the interactions between them [62]. These interactions can be between different phenolic compounds or between phenolic compounds and other molecules that contribute to antioxidant activity (such as reducing carbohydrates, carotenoids, tocopherols and terpenes, among others). These interactions can be synergistic or antagonistic, depending on the case [61]. Therefore, depending on the selectivity of the solvent to extract these molecules, different results will be obtained in the analyzes of antioxidant activity and TPC, which means that higher values in TPC are not necessarily reflected in an increase in antioxidant activity and viceversa. In addition to what was previously discussed, as mentioned in Sect. 3.1, the different methods to determine both TPC and antioxidant activity in general provide complementary information, because each method evaluates different mechanisms of action of phenolic compounds. All of this could help to understand why the water content in the NADES did not significantly affect the TPC but it did affect the antioxidant activity of the extracts obtained in the present work.

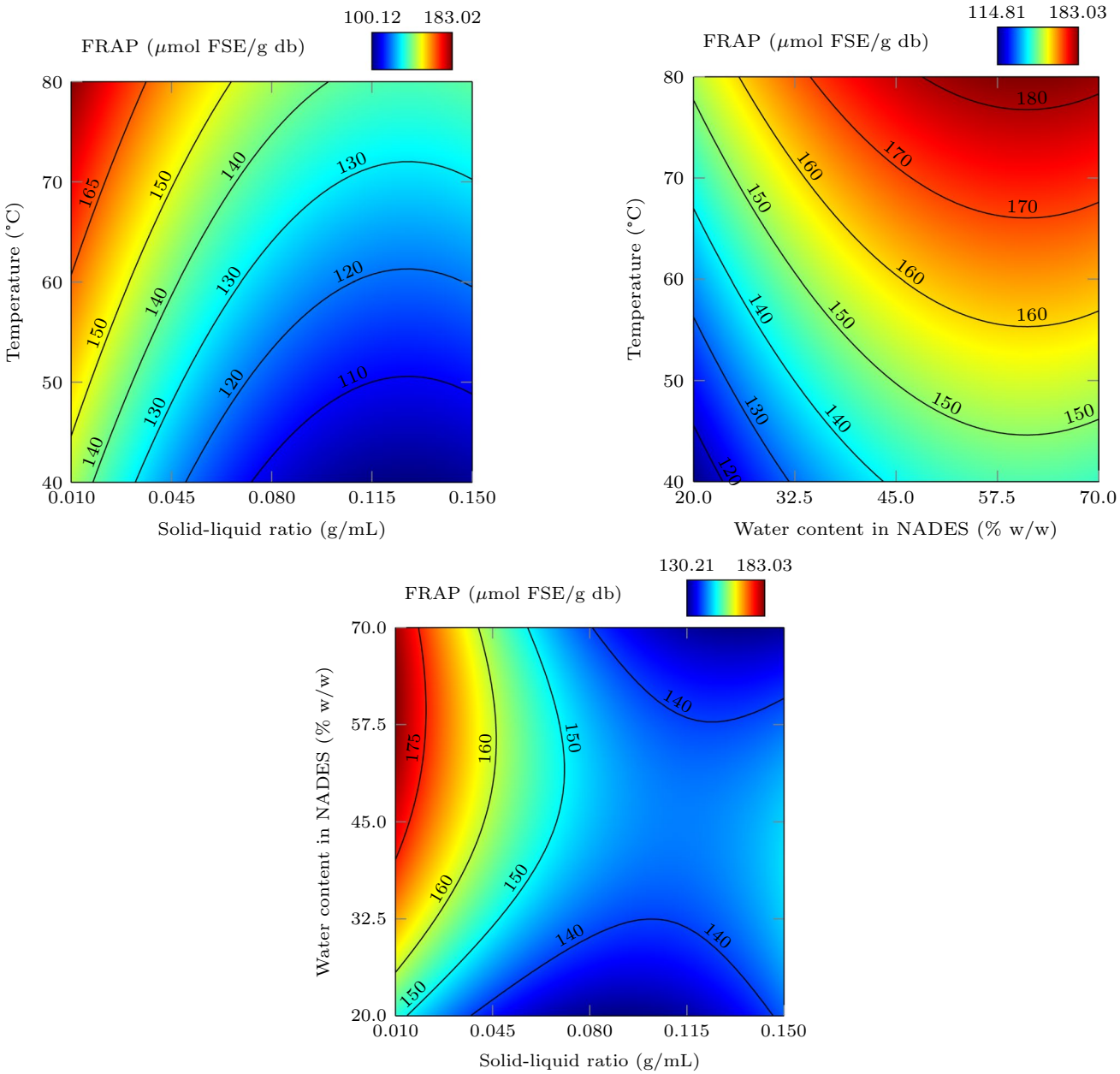

Fig. 4 Contour plots representing the antioxidant activity measured with FRAP of extracts from OP using La-Gc as extraction solvent, as a function of solid–liquid ratio, temperature and water content in NADES

3.3 Optimization of the extraction conditions and verification of the models

In Fig. 6 it is shown the contour plot representing the overall desirability as a function of solid–liquid ratio and water content in NADES at an extraction temperature of 80 °C. This temperature corresponds to the maximum values obtained for TPC and antioxidant activity, which makes the desirability function take higher values. An area is observed where the overall desirability function takes maximum values close to 1, which corresponds to a solid–liquid ratio close to 0.010 g/mL and a water content in NADES between 50–70% (w/w). This is in agreement with what was analyzed in Sect. 3.2, where the effect of extraction conditions on each of the individual responses was discussed. Accordingly, the optimal extraction conditions that maximize the TPC and the antioxidant activity determined by FRAP and DPPH were found at a temperature of 80 °C, water content in NADES of 68% (w/w) and solid–liquid ratio of

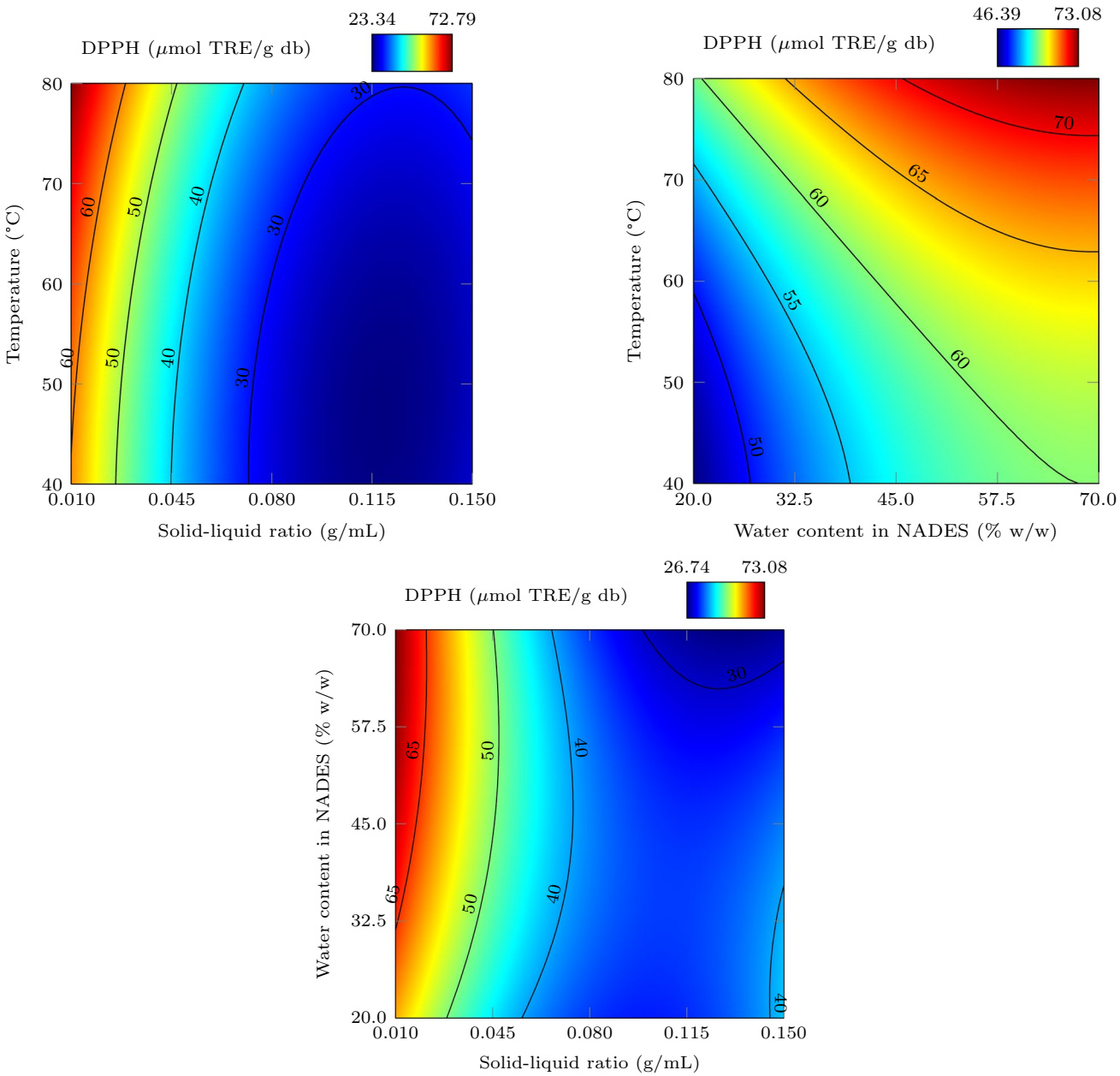

Fig. 5 Contour plots representing the antioxidant activity measured with DPPH of extracts from OP using La-Gc as extraction solvent, as a function of solid–liquid ratio, temperature and water content in NADES

0.014 g/mL. As previously mentioned, although there is an area with similar values of the overall desirability function for a certain range of water content in NADES, the highest value of this variable in that area was chosen as the optimal value, since it implies working with a higher water content in relation to the content of lactic acid and glucose.

To verify the models obtained through RSM, three extractions were performed under the optimum conditions obtained previously. In Table 6 it is presented the experimental results of these extractions along with the values predicted by the models under the optimal extraction conditions. As can be seen, the experimental values of the three responses are within the confidence intervals of the values predicted by the models (with 95% confidence level), which indicates a good degree of prediction of the models obtained in this work under the extraction conditions tested.

On the other hand, Table 6 presents the experimental data of TPC and antioxidant activity using conventional solvents under the same optimal extraction conditions used for the case of La-Gc as solvent (with the exception of the extraction with 70% (v/v) ethanol, where a temperature of 78 °C was used, corresponding to the boiling point of the

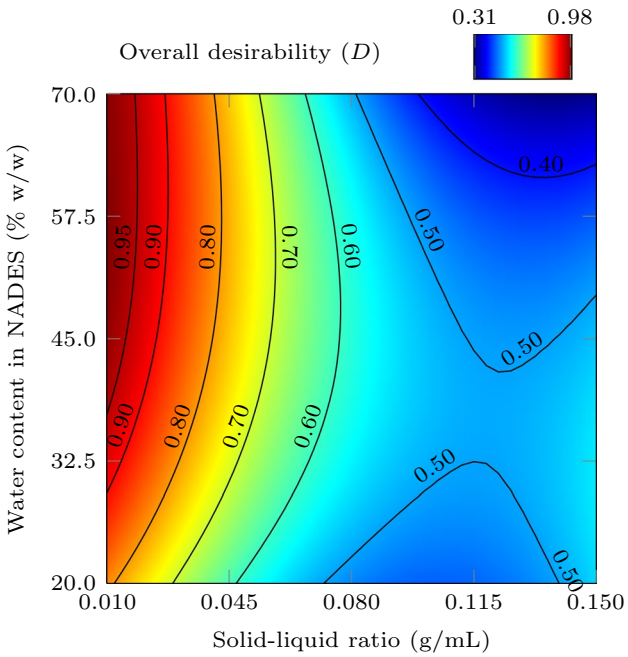

Fig. 6 Contour plot representing the overall desirability as a function of solid–liquid ratio and water content in NADES at temperature of 80 °C

solvent). Significantly higher TPC values and antioxidant activity were achieved for the extracts obtained from La-Gc compared to the use of water and 70% (v/v) ethanol as solvents. As a result, the suggested methodology demonstrated superior extraction characteristics using an environmentally friendly solvent. However, it is necessary to highlight that the comparison with conventional solvents was carried out in a single extraction condition. Further tests should be performed to expand the extraction conditions using conventional solvents and thus improve the comparison between these solvents and La-Gc under the optimal extraction conditions found in the present work.

The total phenols content obtained in the present study using La-Gc as solvent under the optimum extraction conditions (15.56 mg GAE/g db) are under the same order of magnitude than the values reported by Fernández-Prior et al. [58] (used different NADES in conventional extraction from OP, obtained TPC values between 11 and 20 mg GAE/g db at 90 °C for extraction times of 30 and 120 min, respectively) and Pontes et al. [63] (used different NADES in conventional extraction from OP, obtained TPC values between 9 and 25 mg GAE/g db at temperatures between 33 and 60 °C for three hours of extraction time). Chanioti and Tzia [8] also reported values of TPC of extracts obtained from OP using different NADES as solvents but with other technologies different than the used by conventional extraction (ultrasonic or microwave assisted extraction, high pressure assisted extraction and homogenate assisted extraction). Their values ranged from 6 to 34 mg GAE/g db, which are similar to the TPC values found in this work. Dauber et al. [31] performed the extraction of phenolic compounds with antioxidant activity from OP by extraction with supercritical CO_2 and ethanol as co-solvent. Despite the fact that it is a different extraction technology from the one used in the present work, it is interesting to compare their results since they used OP of a very similar origin

Table 6 Experimental and predicted values by the models of TPC and antioxidant activity at temperature of 80 °C, water content in NADES of 68% (w/w) and solid–liquid ratio of 0.014 g/mL

	TPC	FRAP	DPPH
La-Gc - Predicted	15.25 ± 0.64	178.79 ± 10.70	70.05 ± 6.86
La-Gc - Experimental	15.56 ± 0.55^a	178.14 ± 4.57^a	72.75 ± 4.10^a
70% (v/v) ethanol	14.17 ± 0.08^b	153.26 ± 1.05^b	55.10 ± 0.31^b
Water	10.79 ± 0.24^c	108.29 ± 4.62^c	50.76 ± 0.31^b

TPC: total phenols content (mg GAE/g db); FRAP: antioxidant activity (μmol FSE/g db); DPPH: antioxidant activity (μmol TRE/g db). Experimental values correspond to mean ± standard deviation of three replicates. Confidence intervals of predicted values correspond to 95% confidence level. Values with different letters are significantly different (Tukey test, $p < 0.05$)

Comparison with conventional solvents at the same extraction conditions (with the exception of the extraction with 70% (v/v) ethanol, where a temperature of 78 °C was used)

(Maldonado, Uruguay) and variety (Arbequina). It is noteworthy to mention that the values of TPC reported in the present work are 20-fold higher than those reported by Dauber et al. [31] (optimum value of 0.76 mg GAE/g db). This highlights the suitability of the present extraction method using La-Gc as solvent to obtain phenolic compounds with antioxidant activity from OP.

With respect to antioxidant activity, results obtained in this work are in line with the reported by Nunes et al. [64], who carried out extractions with ethanol-water 80% (v/v) in a shaking water bath at room temperature for 3 h from OP of different varieties. They reported FRAP values in the range of 111–233 μmol FSE/g db and DPPH values in the range of 50–85 μmol TRE/g db. Quero et al. [65] performed extractions from OP using water and ethanol water 50% (v/v) by ohmic heating and by extraction in a stirred water bath, in both cases at 80 °C, 30 min and a solid–liquid ratio of 0.1 g/mL. They reported values of FRAP of 80 μmol FSE/g for water extracts and 130–150 μmol FRE/g for ethanol-water extracts. Ribeiro et al. [66] reported values of DPPH ranging from 41 to 106 μmol TRE/g db in extracts obtained from OP using methanol in an orbital shaker at room temperature and 60 min with solid–liquid ratio of 0.01 g/mL.

3.4 Hydroxytyrosol content

Hydroxytyrosol, among the primary phenolic compounds found in olive pomace, has garnered significant attention over time. This particular molecule exhibits potent antioxidant activity, as well as anti-inflammatory and antimicrobial properties. Consequently, the cosmetics and pharmaceutical industries show great interest in harnessing the potential of this compound. Additionally, researchers have also investigated its impact on the quality of food, including its effects on chemical composition, nutritional value, and sensory characteristics [5, 67]. This highlights the importance of quantifying the hydroxytyrosol content in the extracts obtained from OP.

Table 7 shows the hydroxytyrosol content in the extracts obtained from La-Gc under the optimal extraction conditions determined in Sect. 3.3, together with the respective values of the extracts obtained from conventional solvents under the same extraction conditions. As can be seen, there are no significant differences in the hydroxytyrosol content using 70% (v/v) ethanol or water as solvents, while the content of this molecule is significantly higher in the case of the extracts obtained with La-Gc (almost 2 times higher).

Different hydroxytyrosol contents of extracts obtained from OP under extraction conditions similar to those reported in this work have been published. Fernández-Prior et al. [58] reported hydroxytyrosol contents between 0.33 and 0.50 mg/g db using conventional extraction with NADES as solvents at temperature of 90 °C and extraction times of 30–120 min (they reported higher contents but using an autohydrolysis process at higher temperatures). Panić et al. [21] reported hydroxytyrosol contents of 0.20 mg/g db using microwave and ultrasound assisted extraction with NADES as solvent at extraction time of 10 min (they did not informed the extraction temperature). Fernández et al. [19] reported hydroxytyrosol contents of 0.11 mg/g db using ultrasound assisted extraction and La-Gc 5:1 molar with 15% water content as solvent at temperature of 40 °C and extraction time of 60 min. The same investigation group reported hydroxytyrosol contents ranging between 1.0 and 3.0 mg/g db using the same extraction system and conditions but with different OP [53]. Chanioti and Tzia [8] reported hydroxytyrosol contents between 0.33 and 1.40 mg/g db using microwave and ultrasound assisted extraction with different NADES as solvents at temperatures between 40 and 60 °C and extraction times of 30 min. They informed higher hydroxytyrosol contents (up to 6.0 mg/g db) but using other kind of technologies (high pressure assisted extraction and homogenate assisted extraction).

Table 7 Hydroxytyrosol content of extracts from OP at temperature of 80 °C and solid–liquid ratio of 0.014 g/mL using La-Gc with water content of 68% (w/w)

Solvent	Hydroxytyrosol content
La-Gc (water content: 68% (w/w))	1.24 ± 0.05^a
70% (v/v) ethanol	0.66 ± 0.01^b
Water	0.62 ± 0.02^b

Hydroxytyrosol content: mg/g db. Values correspond to mean ± standard deviation of three replicates. Values with different letters are significantly different (Tukey test, $p < 0.05$)

Comparison with conventional solvents at the same extraction conditions (with the exception of the extraction with 70% (v/v) ethanol, where a temperature of 78 °C was used)

4 Conclusions

The present work allowed to evaluate the use of different NADES as green solvent in the solid–liquid extraction of phenolic compounds with antioxidant activity from OP produced in Uruguay through a technologically simple process that could be easily adapted to the national production scale. In this sense, several of the NADES studied showed good characteristics for extracting phenolic compounds with antioxidant activity. In particular, La-Gc with a 5:1 molar ratio was selected as the best solvent among the tested NADES.

TPC and antioxidant activity of the extracts were significantly affected by the tested variables of the extraction process: temperature, water content in NADES and solid–liquid ratio. The mathematical models that describe the relationships between the different operating conditions tested and the response variables of the extraction process (TPC, FRAP and DPPH) were obtained. From these models, the optimal extraction conditions that maximized the TPC and antioxidant activity were determined: 80 °C, 68% (w/w) of water content in NADES and solid–liquid ratio of 0.014 g/mL. TPC and antioxidant activity of NADES extracts under optimal extraction conditions were significantly higher than those corresponding to the extracts obtained by using conventional solvents (water and ethanol-water mixture) under the same extraction conditions. This highlights the suitability of the present extraction method using La-Gc as solvent to obtain phenolic compounds with antioxidant activity from OP.

Further research should be carried out in order to study if it is possible to directly apply the extracts obtained by the methodology used in this work to food and cosmetic products, taking advantage of the fact that the components of NADES (lactic acid and glucose) are widely used as additives or food ingredients; or if a previous stage of separation is needed in order to obtain a purified extract of phenolic compounds with antioxidant activity.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Competing interests The authors declare no competing interests.

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