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Proceeding Paper

Natural Deep Eutectic Solvents as the Main Solvents for the Extraction of Total Polyphenols from Orange Peel †

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Abstract: The extraction of bioactive compounds is generally carried out using organic solvents, although they are very harmful to the environment. The present study focuses on the extraction of bioactive compounds using natural deep eutectic solvents (NADESs), which are novel, green, and low-melting-point solvents. In this work, we seek to optimize the extraction process of total polyphenols from orange peel using four types of NADESs with different water concentrations, solid–liquid ratios, and extraction times. The results show that the best percentage of NADESs were as follows: 10, 30, and 50%; the solid–liquid ratio differs depending on the compound, and the optimal extraction time is generally estimated to be 30 min.

Keywords: natural deep eutectic solvents; orange peel; total polyphenol content

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1. Introduction

Every year, the food industry generates a considerable amount of waste, particularly in the citric sector, where 18 percent of the citric fields are used for industrial processes, resulting in enormous amounts of garbage. This waste has a high biological value and may be utilized for functional food. Plant-based foods include a high concentration of bioactive, phytochemical, or phytonutrient components, which are divided into the following four categories: nitrogenous chemicals, sulfurated compounds, terpenes, and phenolic compounds [1].

In recent years, the concept of green chemistry and green economy has grown. Traditionally, the use of organic solvents, such as methanol or dichloromethane, as well as non-green extraction methods for the extraction of bioactive compounds from plants and vegetables has generated a large amount of non-ecofriendly solvent residues and high-energy use [2].

Supercritical fluids (SCFs), ionic liquids (ILs), and deep eutectic solvents (DESs) have recently been employed as alternatives to organic solvents for the extraction of bioactive compounds. Natural deep eutectic solvents (NADESs) are DESs made of small natural compounds, such as sugars, organic acids, or amino acids. Their molecular interactions and hydrogen bonding give their physiochemical properties, including a low fusion point and high viscosity, making them a suitable alternative to organic solvents [3].

Orange peel is an agri-food industry byproduct with high biological value since it is a portion of the fruit that contains substantial levels of bioactive compounds that act by delaying oxidation, preventing the presence of insects, and preserving the fruit from mechanical harm [4].

This research aims to optimize the extraction procedure for total polyphenol content (TPC) from orange peel using NADESs.

2. Materials and Methods

2.1. Raw Material

Orange peels were obtained from the Navel variety, donated by a local agricultural cooperative. The peels were removed from the pulp, crushed with a blender, and used immediately.

2.2. Chemical and Reagents

Choline chloride (≥98%), D-(−)-fructose (≥99%), and Folin–Ciocalteu reagent were purchased from Sigma-Aldrich (Steinheim, Germany). Glycerol was obtained from Glentham Life Science (Corsham, UK). DL-malic acid (≥98%) was purchased from Thermo Fisher (Kendel, Germany). Citric acid (≥99.9%) and anhydrous sodium carbonate (Na_2CO_3) were purchased from VWR Chemicals (Leuven, Belgium). L-Proline was purchased from Guinama (Spain). Betaine was obtained from Fluorochem (Hadfield, UK).

2.3. Preparation of Natural Deep Eutectic Solvents

NADESs were prepared following the method described by Dai et al. (2015) [5], with modifications. NADESs were prepared by mixing the reagents with different molar ratios (according to their molar mass) and heating and stirring at 60–80 °C in a water bath until a liquid was formed.

Four combinations of NADESs were prepared, where Choline Chloride (ChChl), Betaine (Bet), and the amino acid L-Proline (LP) acted as Hydrogen Bond Acceptors (HBAs), and the sugar Fructose (Fruc), the two organic acids Malic Acid (MA) and Citric Acid (CA), and the polyalcohol Glycerol (Gly) acted as Hydrogen Bond Donors (HBDs) (see Table 1). They were mixed with different amounts of water (NADESs in 10%, 20%, 30%, 40%, 50%, 75%, and 85% of water) to reduce the viscosity.

Table 1. Materials and molar ratios of the studied NADESs.

Acronym	HBA	HBD	Molar Ratio
ChChl:Fruc	Choline Chloride	Fructose	1.9:1
ChChl:Gly	Choline Chloride	Glycerol	1:2
Bet:CA	Betaine	Citric Acid	1:1
LP:MA	L-Proline	Malic Acid	1:1

HBA, Hydrogen Bond Acceptor. HBD, Hydrogen Bond Donor.

2.4. Extraction Procedure

The bioactive compounds were determined in the orange peel–NADES extract, considering the concentration (solid–liquid ratio), the type of NADES, the percentage of water, and the extraction time. The extraction was carried out by magnetic stirring at 45 °C. Then, the samples were centrifuged in a 5810 R centrifuge (Eppendorf, Germany) at 5 °C, 18× *g* for 10 min. The supernatant was stored and used for the determination of the TPC. The results were optimized using the Response Surface Methodology (RSM) to establish the best conditions for the total polyphenol extraction. The optimization was carried out following the method proposed by Derringer and Suich (1980) [6]. The geometrical mean of the individual functions was calculated by combining all of the individual functions obtained for each response. This approach is more appropriate if the desirability value is close to the unit [7].

2.5. Determination of the Total Polyphenol Content by UV–Vis Spectroscopy

The TPC was determined using the method described by Singleton and Rossi (1965) [6]. A total of 100 µL of the sample was mixed with 3 mL of sodium carbonate (2%, *m*/*v*) and 100 µL of Folin–Ciocalteu reagent (1:1, *v*/*v*). A gallic acid calibration curve was constructed

under the same conditions as the samples. Once the reaction was over (1 h), the absorbance was measured at 765 nm (Perkin Elmer ®, Boston, MA, USA). The TPC was expressed in milligrams of gallic acid equivalent (GAE) per 100 g of the dry weight of the orange peel.

2.6. Statistical Analyses

The optimization results were generated using an RSM (Design-Expert 8.0 for Windows®) (Stat-Ease, Minneapolis, MN, USA). A Box–Behnken design was used with the following three independent variables: X_1, (solid–liquid ratio), X_2 (percentage of NADES in water), and X_3 (extraction time) (shown in Table 2). The mean differences were analyzed by a one-way analysis of variance (ANOVA) to compare two or more values to check if the differences were statistically significant. A post-hoc Tukey's test was performed using the SPSS® 26.0 for Windows® software (SPSS, Chicago, IL, USA). The differences were considered significant at $p < 0.05$. All samples were performed in triplicate, and the data are presented as mean ± SEM.

Table 2. Coded levels of the independent variables.

Independent Variable		Level		
		−1	**0**	**+1**
Solid/liquid ratio	X_1	5	15	25
NADES (%, *v*/*v*)	X_2	10	50	85
Extraction time (min)	X_3	5	15	30

Independent variables of the study.

The optimization plots were generated with the Design-Expert 8.0 for Windows® (Stat-Ease, Minneapolis, MN, USA).

3. Results and Discussion

In this work, four NADESs were optimized for the TPC extraction using a Box–Behnken design, as shown in Table 3. The optimization model chose one of the studied NADESs (ChChl:Fruc), and the extraction yields for these NADESs were obtained. The ANOVA showed *p* values < 0.0001, indicating that the model selected was highly significant.

Table 3. Box–Behnken design with the independent variables and response data.

Runs	Extraction			Total Polyphenol Content (mg GAE/100 g DW)			
	X_1	X_2	X_3	**LP:MA**	**ChChl:Fruc**	**ChChl:Gly**	**Bet:CA**
1	15	50	10	2804.7 ± 146.6	1045.4 ± 73.6	296.3 ± 22.6	1767.4 ± 221.5
2	25	10	15	2341.5 ± 149.3	1688.0 ± 107.0	512.3 ± 3.3	1711.7 ± 117.5
3	5	50	5	2029.4 ± 57.5	4128.3 ± 109.0	535.2 ± 32.5	1445.9 ± 47.8
4	15	30	15	3582.8 ± 506.3	1503.2 ± 32.2	611.4 ± 13.8	1889.4 ± 181.9
5	5	20	20	ND	3337.5 ± 339.4	1706.1 ± 125.7	ND
6	25	30	5	2569.1 ± 59.4	913.8 ± 76.6	177.4 ± 15.7	1254.7 ± 136.6
7	10	50	30	3712.2 ± 1222.6	7577.3 ± 385.1	596.1 ± 16.3	3004.4 ± 238.1
8	15	30	15	3079.6 ± 221.3	1482.8 ± 80.0	609.7 ± 23.7	1852.6 ± 87.2
9	15	30	15	4334.1 ± 441.5	1360.8 ± 37.6	649.9 ± 53.1	1698.9 ± 52.9
10	15	10	30	2130–6 ± 43.3	3220.8 ± 245.0	1088.2 ± 20.2	1630.3 ± 47.9
11	25	30	5	2645.8 ± 50.3	778.9 ± 24.1	190.2 ± 6.6	1194.1 ± 183.9
12	25	10	30	2598.8 ± 133.6	3241.5 ± 40.3	631.4 ± 15.1	1713.4 ± 243.7
13	5	20	20	ND	3637.5 ± 118.2	1717.0 ± 56.4	ND
14	10	10	5	2187.8 ± 92.8	2744.4 ± 768.2	927.2 ± 9.5	1317.2 ± 76.7
15	5	40	20	ND	1480.6 ± 72.4	266.1 ± 13.0	ND
16	25	30	30	4121.5 ± 231.1	515.7 ± 10.3	285.2 ± 16.9	1657.4 ± 93.0

Table 3. *Cont.*

Runs	Extraction			Total Polyphenol Content (mg GAE/100 g DW)			
	X_1	X_2	X_3	LP:MA	ChChl:Fruc	ChChl:Gly	Bet:CA
17	25	50	15	1161.1 ± 11.2	354.9 ± 7.8	214.4 ± 10.0	694.1 ± 70.4
18	20	10	5	2134.5 ± 186.4	729.9 ± 11.1	438.8 ± 14.3	1318.4 ± 9.7
19	15	30	15	4706.2 ± 592.9	1773.3 ± 32.1	594.4 ± 15.3	1742.2 ± 142.3
20	5	30	5	486.7 ± 14.7	2563.9 ± 10.8	944.7 ± 36.6	1455.4 ± 63.0
21	15	75	10	3208.8 ± 573.9	55.7 ± 1.2	27.9 ± 1.2	1458.3 ± 194.0
22	5	75	5	1754.8 ± 79.0	28.2 ± 1.8	16.5 ± 1.4	1090.2 ± 53.2
23	10	75	15	3104.5 ± 68.7	114.4 ± 14.4	87.2 ± 109.1	1707.0 ± 99.4
24	25	75	15	2924.7 ± 214.1	158.9 ± 7.9	35.1 ± 3.0	1199.4 ± 73.9
25	15	85	10	3643.2 ± 279.9	103.8 ± 12.8	37.8 ± 10.0	ND
26	5	85	5	1581.4 ± 87.1	41.3 ± 4.9	216.1 ± 14.3	ND
27	10	85	30	3232.1 ± 308.2	68.8 ± 13.3	212.2 ± 18.5	ND
28	25	85	15	3895.8 ± 213.0	188.6 ± 34.8	115.8 ± 3.3	ND

X_1, solid–liquid ratio; X_2, %NADES; X_3, extraction time; ND, not detected; LP:MA, L-Proline:Malic Acid; ChChl:Fruc, Choline Chloride:Fructose; ChChl:Gly, Choline Chloride:Glycerol; Bet:CA, Betaine:Citric Acid; GAE, gallic acid equivalent. DW, dry weight.

3.1. NADESs Mixed with Water

To decrease the viscosity, each NADES was mixed with different amounts of water. Dai et al. [5] observed that at >50% of water, the hydrogen bonds may break, giving lower extraction yields. In addition, NADESs mixed with more than 50% of water are considered aqueous solutions and not eutectic mixtures anymore [8]. The extraction yields for each NADES with a different amount of water content are shown in Figure 1. In the cases of ChChl:Gly and LP:MA, the best extraction yields were observed at a lower NADES content, in agreement with the results given by Mouratoglou et al. [9], studying the extraction of polyphenols with 90% aqueous NADES. Moreover, Benvenutti et al. showed that high water content does not hinder NADESs from being effective solvents for the extraction of bioactive substances and would reduce costs [10].

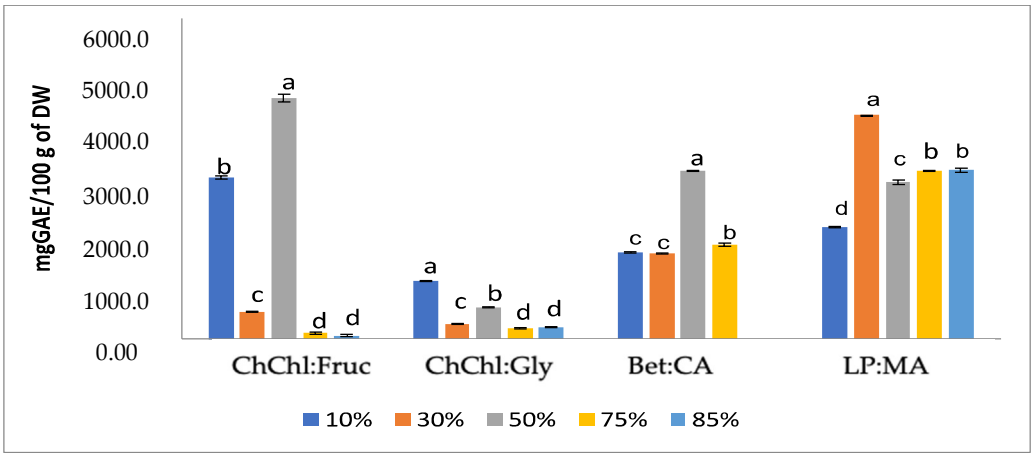


Figure 1. Total phenolic content in NADES extracts with different amounts of water. ChChl:Fruc, Choline Chloride:Fructose; ChChl:Gly, Choline Chloride:Glycerol; Bet:CA, Betaine:Citric Acid; LP:MA, L-Proline:Malic Acid. a–d: the different letters indicate that there are statistically significant differences ($p < 0.05$).

ChChl:Fruc and Bet:CA showed the best extraction yields at 50% of water, considering these NADES combinations of eutectic mixtures.

3.2. Optimization

To optimize the extraction of the TPC, an ANOVA was performed (Supplementary Material), and an RSM established the best conditions for the extraction. The optimization was done separately for each NADES to compare the extraction conditions. Table 4 shows the best conditions for the solid–liquid ratio and the extraction time for the extraction of the TPC with each studied NADES. The optimum extraction ratio was almost the same in three of the NADES but in LP:MA, and the optimum extraction time would be between 23 and 30 min. The extraction yield of LP:MA is one of the highest, which could be due to the fact that, for its polarity, it is an optimum NADES for the extraction of TPC [11].

Table 4. Optimum conditions for the extraction.

Acronym	Ratio (solid/liquid)	Extraction Time (min)	Max (mg GAE/100 g DW)	Desirability
ChChl:Fruc	5.0	30.0	6530.8	0.8
ChChl:Gly	5.2	23.3	1833.5	1.0
Bet:CA	6.0	28.5	3218.8	1.0
LP:MA	16.7	29.0	5389.1	1.0

LP:MA, L-Proline:Malic Acid; ChChl:Fruc, Choline Chloride:Fructose; ChChl:Gly, Choline Chloride:Glycerol; Bet:CA, Betaine:Citric Acid. GAE: gallic acid equivalent. DW: dry weight.

Having noticed that, ChChl:Fruc and Bet:CA showed the best extraction yield at a low percentage of water and being considered eutectic mixtures, they were optimized. In order to show just the significant interactions between the two variables on the response values, 3D-response surface plots were created. They show the interactions between the process variables for the TPC (Figure 2), indicating the best extraction conditions, and each plot shows all of the interactions of the factors that are statistically significant ($p < 0.005$). For the ChChl:Fruc, Figure 2 shows that for a higher percentage of NADES, a lower ratio is needed to increase the extraction. For the Bet:CA, the lower extraction time presents a higher extraction yield. At the same time, the equation for the method validation is shown in (1). For the optimization process, NADES 50% water was used. Sugar- and organic acid-based NADES are viscous, colorless, and transparent mixtures at room temperature; their matrix could be a good net for polyphenol extraction.

$$\begin{aligned} \text{TPC} = 678.72908 \quad & +648.85063\ X_1 + 142.90774\ X_2 - 1458.53645\ X_3 \\ & +16.18223\ X_1\ X_3 - 9.13316\ X_2 - 14.22819\ X_2^2 + 97.14280\ \text{X}^2 \\ & -0.214174\ X_1\ X_2\ X_3 + 0.058485\ X_1\ X_2^2 - 0.508917\ X_1\ X_3^2 \\ & -0.025242\ X_2^2\ X_3 + 0.378015\ X_2\ X_3^2 - 1.89564\ X_3^3 \end{aligned} \quad (1)$$

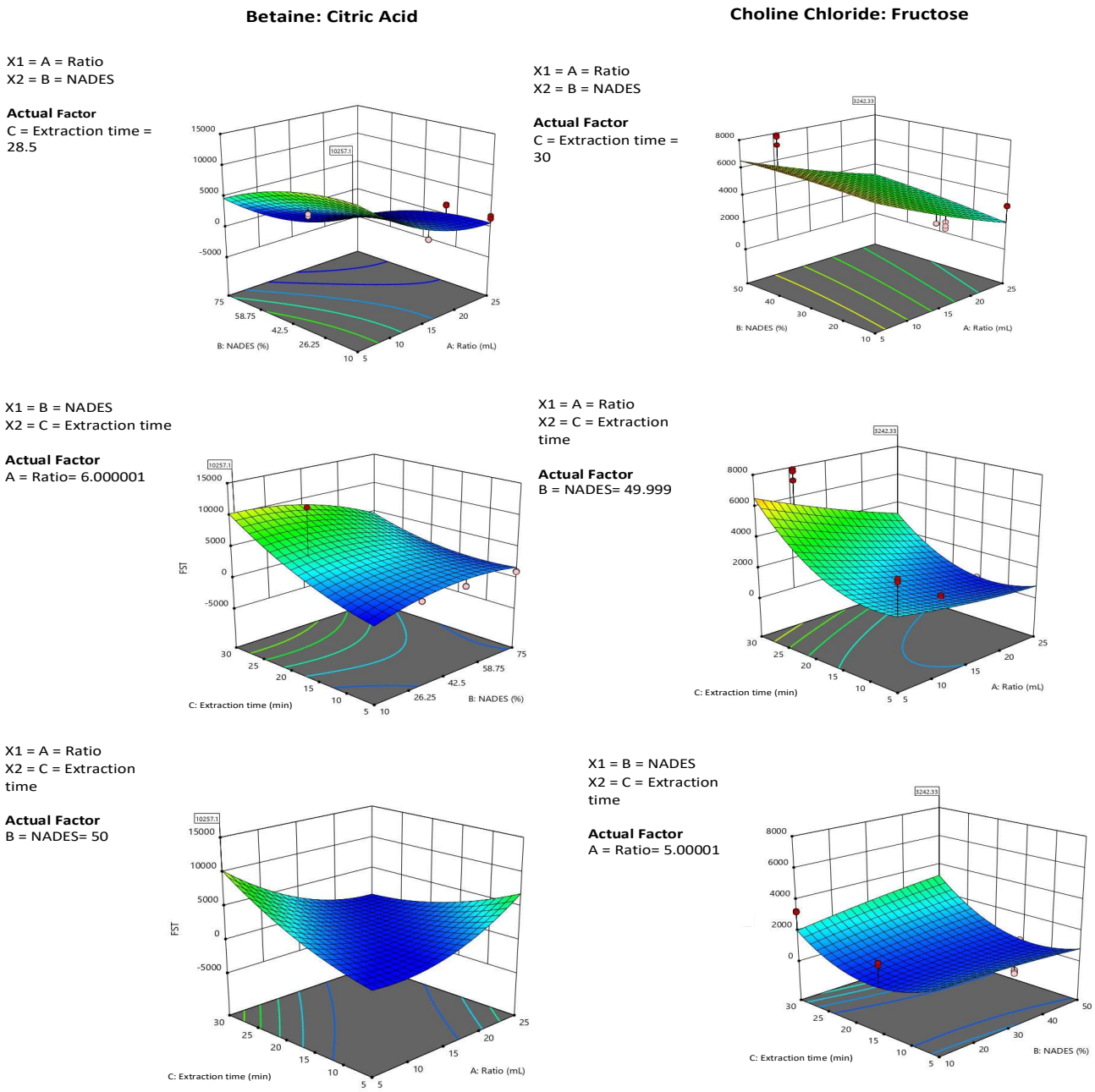


Figure 2. Optimization plots. ChChl:Fruc Choline, Chloride:Fructose. Bet:CA, Betaine:Citric Acid.

4. Conclusions

The extraction of the total polyphenol content with NADES was viable. Depending on the type of NADES used, the best percentage of NADESs in water was considered an eutectic mixture or an aqueous solution. ChChl:Fruc and Bet:MA are the eutectic mixtures considered optimum for the extraction of the TPC. NADES turned out to be a good alternative to common organic solvents for the extraction of TPC.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/Foods2021-11043/s1, Table S1: ANOVA Proline:Malic Acid; Table S2: ANOVA Betaine: Citric Acid; Table S3: ANOVA Choline Chloride: Glycerol; Table S4: ANOVA Choline Chloride: Fructose.

Author Contributions: C.G.-U.: Investigation, formal analysis and Writing-Original draft preparation; A.V.-O.: Investigation, Methodology and Formal analysis; A.P.-S.: Investigation; J.B.: Writing-

Reviewing and Editing; D.L.-M.: Writing-Reviewing and Editing; M.J.E.: conceptualization, supervision and funding acquisition; A.F.: Writing-Reviewing. All authors have read and agreed to the published version of the manuscript.

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