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Green Extraction of Flavonoids from Orange Peels Using Deep Eutectic Solvents †

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Abstract: The aim of this study was to optimize and compare four different natural deep eutectic solvents (NADES) for the extraction of flavonoids in orange peels (Navel cultivar) from Valencia (Spain). Four NADES systems with two components were obtained in their corresponding molar ratios. Three independent variables were used for the optimization: solid-liquid ratio, extraction time and the percentage of NADES in water. The results showed the highest extraction was with choline chloride: fructose (NADES-1) with 50% water content, a solid–liquid ratio of 1:25 and extraction time of 23 min. The results demonstrate that the use of NADES is an efficient and ecofriendly alternative to extracted flavonoids from orange peels.

Keywords: flavonoids; extraction; green solvents; natural deep eutectic solvents; oranges

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1. Introduction

Global orange production is estimated at around 115.5 million tons per year and 50% of this weight accounts for its by-products, which generate around 3 million tons per year of waste [1]. By-products include peel (flavedo and albedo), pulp and seeds, which are sources of high value-added compounds [2]. Orange peel is a chemically complex substrate containing an important variety of bioactive compounds, including fermentable sugars, carbohydrate polymers, flavonoids, polyphenols, vitamins, essential oils and carotenoids [3]. Flavonoids are one of the most important metabolites in plants and they have important biological functions. Conventional extraction of these bioactive compounds has been carried out with organic solvents but most of them are toxic and pollute the environment. As an alternative for organic solvents, natural deep eutectic solvents (NADES) have recently received more attention [4].

NADES are composed of components that exist in nature and act as hydrogen bond acceptors (HBAs) or hydrogen bond donors (HBDs) [5]. NADES have some advantages; they are nonflammable, miscible with water, easily degradable, biocompatible, nontoxic and have high extraction power for different polar substances in plants [6]. The aim of this study was to optimize and compare four different NADES for the extraction of flavonoids in orange peels (Naveline cultivar) from Valencia (Spain).

2. Materials and Methods

2.1. Raw Material

The peels were obtained from orange fruits (Navel cultivar) purchased at a local supermarket (Valencia, Spain). The oranges were washed with distilled water and used

immediately. The orange peels were removed from the pulp and milled with a food grinder and stored at 4 °C for the follow-up experiments.

2.2. Preparation of Natural Deep Eutectic Solvents

Deep eutectic solvents were prepared, according to the method of Dai et al. (2015) [7], with some modifications. NADES were prepared by mixing the reagents in specific molar ratios [8] and then stirring the mixture at 60–80 °C in a water bath until a transparent liquid formed. Four different NADES systems with two components (HBA and HBD) were obtained, specific ratios with 10, 30, 50, 75 and 85% of NADES in water (*w*/*w*) were placed to obtain liquids at room temperature. Table 1 shows the composition, molar ratios, and codes of the NADES used in this study.

Table 1. Components and molar ratios of the NADES.

No.	Hydrogen Bond Acceptor	Hydrogen Bond Donor	Molar Ratio
NADES-1	Choline chloride	Fructose	1.9:1
NADES-2	Choline chloride	Glycerol	1:2
NADES-3	Proline	Malic acid	1:1
NADES-4	Betaine	Citric acid	1:1

2.3. Extraction Procedure

Orange peel samples were placed in a beaker with NADES solvent. For the screening of the optimum solvent, the four NADES were mixed in a solid–liquid ratio of 1:10 g/mL for 30 min. The extraction was carried out by magnetic stirring and heating. The temperature was 45 ± 5 °C for each sample. The samples were then centrifuged in a 5810 R centrifuge (Eppendorf, Germany) at 5 °C, 3000 rpm for 10 min. The supernatant layer was stored in dark tubes at 4 °C until analysis. After the solvent screening, the response surface methodology (RSM) was applied with the optimal NADES to optimize the extraction conditions for flavonoid extraction. The optimization was performed following the method proposed by Derringer and Suich (1980) [9]. All the individual desirability functions obtained for each response were combined into an overall expression, which is defined as the geometrical mean of the individual functions. The nearer the desirability value to the unit, the more adequate the system (Ross, 1996) [10].

2.4. Total Flavonoid Content

The total flavonoid content (TFC) of the orange peel was determined using the method of Zhishen et al. (1999) [11]. An aliquot of 100 µL of the sample was mixed with 1088 µL of ethanol (30%, *v*/*v*) and 48 µL of sodium nitrite (0.5 mol/L) and vortex. After 5 min of reaction time, 48 µL of aluminum chloride (0.3 mL/L) was added. The sample was able to react for 5 min and 320 µL of sodium hydroxide (1 mL/L) was added and vortexed again. The absorbance was measured at 510 nm using a spectrophotometer UV/VIS (Perkin Elmer® Lambda 365, Lima, OH, USA). The catechin (2 mg/mL) calibration curve was carried out under the same conditions as the samples. The TFC results were expressed in mg of catechin equivalents (CE) per 100 g of dry weight (DW) orange peel.

2.5. Experimental Design

The optimization parameters of the NADES were examined using RSM (Design Expert Software 11.0). A Box–Behnken design was performed with three independent variables of X_1, (liquid–solid ratio), X_2 (% NADESs in water) and X_3 (extraction time). The levels and variables are presented in Table 2.

Table 2. Coded levels of independent variables.

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

2.6. Statistical Analyses

The response surface plots were generated with Design-Expert 8.0 (Stat-Ease, Minneapolis, MN, USA), which was used to design the experimentation along with data analysis. The difference between the mean values was analyzed by the ANOVA test, followed by the post hoc Tukey's test using SPSS (software Version 23) (IBM, Armonk, NY, USA) The significance of the results was assessed at $p \leq 0.05$.

3. Results and Discussion

3.1. Evaluation of NADES Composition on Flavonoid Extraction Efficiency

Four different types of NADES were screened for the extraction of flavonoids from orange peels, resulting in different extraction efficiencies (Figure 1). NADES-1 (Choline chloride–Fructose) was found to be the most effective. Several authors have indicated that the percentage of NADES in water has a significant effect on the extraction efficiency, because the viscosity of the resulting NADES solution can vary with the water content [12,13]. Figure 1 shows that when the content of NADES was 85%, the extraction yield decreased, because flavonoids dissolutions and the penetration in the target matrix are limited by the high viscosity [14,15]. In all cases the extraction with 85% of NADES allowed the lowest flavonoid yields. NADES-1 showed the highest values of flavonoids, and for that reason it was used for the subsequent experiments.

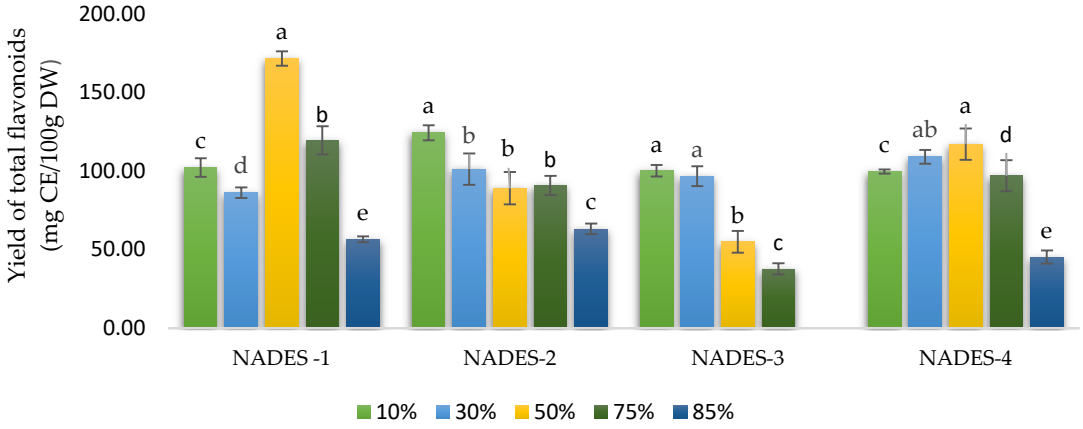

Figure 1. Total flavonoid extraction yields for natural deep eutectic solvents (NADES-1 to NADES-4) according to the percentage of NADES in water, 1:10 solid–liquid ratio and 30 min of extraction. a–e: different letters indicate that there are statistically significant differences ($p < 0.05$).

3.2. Optimization of Flavonoid Extraction by Response Surface Methodology

To optimize the extraction of flavonoids from orange peels a Box–Behnken design was established (Table 3). ANOVA data is summarized in Table 4. The model *p*-value was 0.0060, indicating that the model was highly significant. Table 4 shows that only the percentage of NADES in water has a significant impact in the total yield of flavonoid extraction. The optimum extraction conditions obtained from the RSM analysis for NADES-1 (Choline chloride: Fructose) were 50% of NADES in water, a solid–liquid ratio of 1:25 and an extraction time of 23 min. The optimized conditions showed very close results to those

found in the screening study, where 50% of NADES in water, a solid–liquid ratio of 1:10 and an extraction time of 30 min showed the highest flavonoid yield.

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

Run	Extraction Conditions			Extraction Yield
	X_1	X_2	X_3	TFC
1	15	50	10	51.4 ± 3.5
2	25	10	15	224.4 ± 2.3
3	5	50	5	37.3 ± 1.9
4	15	30	15	147.0 ± 1.2
5	5	20	20	23.6 ± 3.0
6	25	30	5	60.5 ± 4.0
7	10	50	30	103.5 ± 1.7
8	15	30	15	75.2 ± 3.6
9	15	30	15	150.2 ± 3.5
10	15	10	30	102.3 ± 5.9
11	25	30	5	79.0 ± 1.8
12	25	10	30	114.5 ± 5.8
13	5	20	20	26.1 ± 3.2
14	10	10	5	140.9 ± 5.3
15	5	40	20	59.8 ± 2.4
16	25	30	30	86.4 ± 3.4
17	25	50	15	81.8 ± 2.5
18	20	10	5	92.9 ± 5.3
19	15	30	15	95.7 ± 4.5
20	5	30	5	80.4 ± 2.5
21	15	75	10	429.8 ± 1.7
22	5	75	5	316.1 ± 10.4
23	10	75	15	516.8 ± 2.9
24	25	75	15	499.2 ± 2.8
25	15	85	10	29.0 ± 1.8
26	5	85	5	30.0 ± 1.3
27	10	85	15	56.7 ± 1.9
28	25	85	15	41.3 ± 2.5

X_1, X_2 and X_3 represent liquid–solid ratio, % NADES in water and extraction time, respectively. Results were expressed as mean and ± SD.

Table 4. ANOVA for response surface polynomial model of all independent variables.

Source	TFC [a]				
	Sum of Squares	**df**	**Mean Square**	***F*-Value**	***p*-Value**
Model	3.9	9	43,011.8	4.00	0.006 **
X_1	5763.6	1	5763.6	0.53	0.474 ^{ns}
X_2	54,931.9	1	54,931.9	5.11	0.036 *
X_3	113.6	1	113.6	0.01	0.919 ^{ns}
X_1 X_2	43.0	1	43.0	0.01	0.950 ^{ns}
X_1 X_3	2339.6	1	2339.6	0.21	0.647 ^{ns}
X_2 X_3	1088.5	1	1088.5	0.10	0.754 ^{ns}
X_1^2	4240.3	1	4240.3	0.39	0.538 ^{ns}
X_2^2	2666.1	1	2666.1	0.24	0.625 ^{ns}
X_3^2	6922.2	1	6922.2	0.64	0.433 ^{ns}
Residual	1.9	18	10,753.4		
Lack of Fit	1.9	13	14,552.4	16.61	0.003 **
Pure Error	4379.4	5	875.9		
Cor Total	5.8	27			

X_1, X_2 and X_3 represent liquid–solid ratio, % NADES in water and extraction time, respectively; df represents degree of freedom. Level of significance: ** Significant at $p < 0.01$, * Significant at $p < 0.05$, ^{ns} Not significant at $p > 0.05$. [a] TFC: Total flavonoids content from orange peels.

The final polynomial equations in terms of actual factors were:

$$TFC = 114.47 + 23.26X_1 + 74.61X_2 - 3.37X_3 - 1.52X_1X_2 + 21.57X_1X_3 + 11.82X_2X_3 - 28.50X^2_1 + 7.76X^2_2 - 42.02X_3$$

4. Conclusions

In order to design an efficient extraction process, the selection of the most appropriate solvent is a very important step, followed by the optimization of the extraction method, which can be conveniently assisted by RSM. Results demonstrate that the percentage of NADES in the water has a significant impact on the extraction of total flavonoids in orange peels. Our results showed that extraction using NADES is an efficient and ecofriendly alternative to extracting flavonoids from orange peels. Variables studied had significant effects on measured responses.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/Foods2021-10976/s1, Video S1: Green extraction using deep eutectic solvents of flavonoids from orange peels.

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