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

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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 percentage of NADES in water. The result showed the highest extraction was with choline chloride: fructose (NADES-1) with 50% water content, 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 extract 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 the 50% of its weight is accounted for the by-products, which generates around 3 million tonnes 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 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 non-flammable, miscible with water, easily degradable, biocompatible, non-toxic 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 Wei et al., (2015) [8] with some modifications. NADES were prepared by mixing the reagents in specific molar ratios and then stirring the mixture at 60–80 °C in a water bath until a transparent liquid formed. Four different NADESs systems with two components (HBA and HBD) were obtained, specific ratios with 10, 30, 50, 75, 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 NADESs used in this study.

**Table 1.** Components and molar ratios of the NADESs.

| 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. Fort the screening of the optimum solvent, the four NADES were mix in a solid/liquid ratio of 1:10 g/mL for 30 min. The extraction was done by magnetic stirring and heating. The temperature was 45 ± 5 °C for each sample. The samples were then centrifuged in a 5810 R centrifuge (Ependorf, 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.

### 2.4. Total Flavonoid Content

Total flavonoid content (TFC) of orange peel was determined using the method of Zhishen (1999). 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, 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 (Perkin Elmer ® UV/VIS Lambda 365). Catechins (2 mg/mL) calibration curve was carried out under the same conditions as samples. 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 X1, (liquid–solid ratio), X2 (% NADESs in water) and X3 (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*

Response surface plots were generated with Design-Expert 8.0 (Stat-Ease, Minneapolis, MN, USA) was used to design the experimentation along with data analysis. The difference between the mean values were 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. Efficiency Extracting Flavonoids According to Kind of NADES and Percentage of NADES in Water*

Four different types of NADES were screened for their extraction effects (Figure 1). Each NADES have a different extraction efficiency and NADES-1 (Choline chloride/fructose) was found to be the most effective NADES for extraction of flavonoids from orange peels. Chen et al., (2021) and Zannou et al., (2020) [4,9] indicated that the percentage of NADES in water has a significant effect in the extraction efficiency, because the viscosity of the NADES can vary with the water content. In Figure 1 is shown when the content of NADES is 85% the extraction yield decresses, the high viscosity limits the flavonoids dissolutions and the penetration of the solvent in the target matrix [10,11]. In all the cases the extraction with 85% of NADES extracted the lowest yields of flavonoids. Above all, NADES-1 was used for the subsequent experiments.

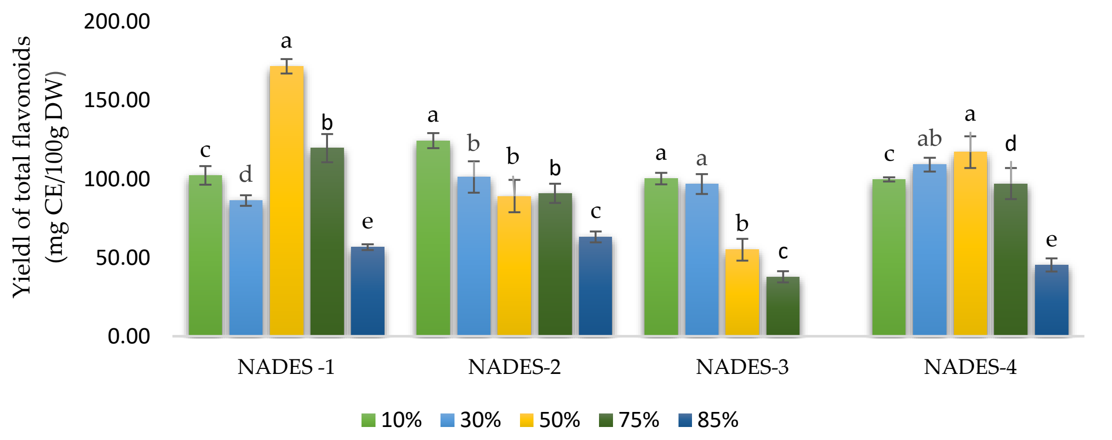


**Figure 1.** Total flavonoid extraction yields for natural deep eutectic solvents (NADES-1 to NADES-4) according to the percentage of NADES in water. * a–e: different letters indicate that there are statistically significant differences ($p < 0.05$).

### *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. The final polynomial equations in terms of actual factors were:

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

**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 | 51.43 ± 3.48 |
| 2 | 25 | 10 | 15 | 224.37 ± 2.34 |
| 3 | 5 | 50 | 5 | 37.27 ± 1.86 |
| 4 | 15 | 30 | 15 | 147.02 ± 1.16 |
| 5 | 5 | 20 | 20 | 23.57 ± 2.96 |
| 6 | 25 | 30 | 5 | 60.54 ± 3.95 |
| 7 | 10 | 50 | 30 | 103.53 ± 1.73 |
| 8 | 15 | 30 | 15 | 75.16 ± 3.59 |
| 9 | 15 | 30 | 15 | 150.18 ± 3.47 |
| 10 | 15 | 10 | 30 | 102.25 ± 5.86 |
| 11 | 25 | 30 | 5 | 79.00 ± 1.76 |
| 12 | 25 | 10 | 30 | 114.51 ± 5.75 |
| 13 | 5 | 20 | 20 | 26.14 ± 3.23 |
| 14 | 10 | 10 | 5 | 140.18 ± 5.33 |
| 15 | 5 | 40 | 20 | 59.81 ± 2.35 |
| 16 | 25 | 30 | 30 | 86.35 ± 3.37 |
| 17 | 25 | 50 | 15 | 81.84 ± 2.5 |
| 18 | 20 | 10 | 5 | 92.88 ± 5.29 |
| 19 | 15 | 30 | 15 | 95.74 ± 4.35 |
| 20 | 5 | 30 | 5 | 80.40 ± 2.48 |
| 21 | 15 | 75 | 10 | 429.81 ± 1.74 |
| 22 | 5 | 75 | 5 | 316.10 ± 10.42 |
| 23 | 10 | 75 | 15 | 516.83 ± 2.85 |
| 24 | 25 | 75 | 15 | 499.21 ± 2.79 |
| 25 | 15 | 85 | 10 | 28.95 ± 1.84 |
| 26 | 5 | 85 | 5 | 30.04 ± 1.27 |
| 27 | 10 | 85 | 15 | 56.66 ± 1.90 |
| 28 | 25 | 85 | 15 | 41.30 ± 2.51 |

$X_1$, $X_2$ and $X_3$ represent liquid-solid ratio, % NADES in water and extraction time, respectively.

**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** | 3.87 | 9 | 43,011.77 | 4.00 | 0.0060 ** |
| $X_1$ | 5763.58 | 1 | 5763.58 | 0.53 | 0.4735 [ns] |
| $X_2$ | 54,931.89 | 1 | 54,931.89 | 5.11 | 0.0364 * |
| $X_3$ | 113.64 | 1 | 113.64 | 0.01 | 0.9193 [ns] |
| $X_1 X_2$ | 42.98 | 1 | 42.98 | 0.01 | 0.9503 [ns] |
| $X_1 X_3$ | 2339.63 | 1 | 2339.63 | 0.21 | 0.6465 [ns] |
| $X_2 X_3$ | 1088.49 | 1 | 1088.49 | 0.10 | 0.7540 [ns] |
| $X_1^2$ | 4240.27 | 1 | 4240.27 | 0.39 | 0.5379 [ns] |
| $X_2^2$ | 2666.07 | 1 | 2666.07 | 0.24 | 0.6246 [ns] |
| $X_3^2$ | 6922.22 | 1 | 6922.22 | 0.64 | 0.4328 [ns] |
| **Residual** | 1.93 | 18 | 10,753.36 | | |
| Lack of Fit | 1.89 | 13 | 14,552.39 | 16.61 | 0.0030 ** |
| Pure Error | 4379.41 | 5 | 875.88 | | |
| **Cor Total** | 5.80 | 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.

### 3.3. Effect of the Studies Variables on Total Flavonoids Content

It can be seen from Table 4 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 software analysis for NADES-1 (Choline chloride/fructosa) were 50% of NADES in water, solid:liquid ratio of 1:25 and extraction time of 23 min.

## 4. Conclusions

The results demonstrate that the percentage of NADES in water has a significant effect in the extraction of total flavonoids in orange peels. Our results showed that extraction using NADES is an efficient and ecofriendly alternative to extract flavonoids from orange peels. Variables studied had significant effects on measured responses.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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