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Proceeding Paper

Assessment of the Use of a Selection of Natural Deep Eutectic Solvents in the Extraction of Polar Bioactive Compounds from Orange Peel †

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Abstract: The reuse of food chain residues is topical. This revaluation can extract bioactive compounds from these residues. However, extraction involves chemicals that cause environmental damage. In the present work, an experimental design with natural deep eutectic solvents (NADES) has been carried out for extracting bioactive compounds from orange peel residues. NADES have a very low environmental impact. The tests were performed with five different NADES, mixed with 70% water. The results were compared with ethanol–water 50%, *v*:*v*, showing that NADES solvents provided better extraction of phenolic compounds and antioxidant capacity. The shelf-life of the extracts was also evaluated, based on the above tests, for 4 weeks, finding significant changes from day 15 of storage at 4 °C.

Keywords: NADES; antioxidant activity; polyphenols; stability; hydrophilic character; orange peel waste

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1. Introduction

In recent years, concern for preserving the environment has become increasingly important. The food industry is trying to find ways to reduce the carbon footprint and pollution of their processes, make better use of food, and reuse residues. With this in mind, the extraction of biofunctional compounds is carried out using organic solvents. These processes have limitations, such as the use of large volumes of solvent, in some cases toxic or harmful to the environment, low selectivity, or the need for purification steps. One of the lines of research of "green chemistry" is to find good sources of biofunctional compounds, such as plant residues, as well as new extraction techniques and less-polluting extractants, minimizing the environmental impact [1,2].

Orange residues obtained from the orange juice industry are one of the most abundant in the food industry. Seventy million tons of oranges are produced annually worldwide, and the peel comprises 40–50% of the total weight of an orange [3].

During the last twenty years, there have been two focuses of study regarding "green solvents". One of them has been on the use of ionic liquids (ILs): formed by a cation and an organic or inorganic anion, joined by ionic bonds. However, their low biodegradability does not make them entirely sustainable, so research has been focused on other areas of

study, deep eutectic solvents (DES) [4]. These have been gaining more importance due to investigations that have been carried out year after year.

The definition given for DES [4] consists of a liquid mixture, formed by two or more compounds, with a melting point that is lower than that of the pure compounds that form it. Thus, two substances that are solid form a liquid phase at their eutectic temperature. Natural deep eutectic solvents are DESs that are formed by natural compounds of low molecular weight, such as sugars, organic acids, and amino acids, among others.

In the present study, whether an increase in the polarity of NADES through its dilution with a high proportion of water is useful and can improve the extraction of polar compounds, such as polyphenols (in the same way that other studies use water as cosolvent to increase solubility) was investigated [5]. If there is a water content greater than 50% in the NADES, it is considered an aqueous solution of its constituents rather than a eutectic mixture. However, another important condition to consider during extraction is viscosity; a high value can hinder the mass transfer between the solute and solvent, which can cause a poor extraction performance. Thus, a higher proportion of water in the NADES would mean a lower viscosity and therefore a better performance [6]. Finally, the shelf-life of NADES extracts has been investigated as a novel approach to this subject.

2. Materials and Methods

2.1. Sample Preparation and Extraction Procedure

The orange samples were bought from supermarkets and grocery stores in the city of Valencia, at the optimum moment of ripening. The type of fruit studied was orange (*Citrus sinensis*, variety Navelina). First, the peels of 6 oranges, each orange had around 200 g of weight, were removed from the pulp and crushed in a blender. Then, 7 g of peel was weighed, and taken for sample analysis; in total 6 samples were used, one for each of the 5 types of NADES used and one more for the mixture of ethanol–water (50%, *v*:*v*). Once each sample was prepared, 70 mL of extractant was added to have a 1:10 solid–liquid ratio, which was placed under magnetic stirring for 20 min to carry out the extraction. Once the time elapsed, the extract was introduced into Falcon® conical tubes and centrifuged for 10 min at 3000 rpm to separate the peel and obtain only the supernatant, which was then introduced into other 50 mL Falcon® conical tubes. These were kept at 4 °C until the time of the different tests. All extractions were carried out in triplicate and the tests were duplicated (total: 36 extracts). The tests were carried out with a sample of fresh peel, but, in parallel, a desiccation test was also carried out in an oven at 100 °C until achieving a constant weight to determine peel water content.

2.2. NADES Preparation

The choice of mixtures was made using a previous bibliographic search, opting for cases where better results were obtained in terms of the extraction of total polyphenols compounds (TPC) and total antioxidant capacity (TAC) in their respective studies (Table 1).

Table 1. NADES selection.

NADES	Molar Ratio	Authors
ChCl: Glu	2:1	Panić et al., 2021 [3]
ChCl: CitAc	2:1	Zhou et al., 2018 [7]
CitAc: Glu	1:1	Xie et al., 2019 [8]
ChCl: Gly	3:1	Mouratoglou et al., 2016 [9]
Glu: Gly	1:1	Panić et al., 2021 [3]

ChCl: choline chloride; Glu: glucose; CitAc: citric acid; Gly: glycerol.

Various options to create NADES (quaternary amine, sugar, organic acid, and glycerol) were used. Their components were choline chloride (ChCl), glucose (Glu), citric acid (CitAc), and glycerol (Gly), and used the same stoichiometric proportions of the original studies. Subsequently, these were heated to between 50 and 90 °C in a water bath to

facilitate melting, and then, a volume of water was added. In the studies, the water content was 30% for each NADES, so when inverting the proportions of water to achieve a more hydrophilic character, 70% was used.

2.3. Followed Protocols

TAC measurements were performed using the DPPH method [10]; meanwhile, measurements of TPC content were quantified using the Folin–Ciocalteau method [11].

2.4. Statistical Analysis

Statistical analysis of the data obtained using the SPSS v.26 program was performed. To compare PFT and CAT of the same extract per day, and between the 6 extracts, a test of homogeneity of variances was carried out using an ANOVA test. A post hoc test of multiple comparisons, HSD Tukey, was used to check for differences between the extracts on a per day basis.

3. Results

3.1. Total Antioxidant Capacity

The TAC estimate was represented by percentage of DPPH inhibition and its evolution throughout the 4 weeks is shown in Table 2.

Table 2. Comparison of total antioxidant capacity for different tested NADES.

Inhibition (%)	Day 1	Day 2	Day 3	Day 8	Day 10	Day 15	Day 17	Day 22	Day 24
ChCl-Gly	41.7 ± 2.9 (b)	47.0 ± 6.5 (b)(f)	52.0 ± 2.2 (b)(c)(f)	46.6 ± 4.2 (b)(d)(e)(f)	48.2 ± 2.3 (b)(e)(f)	**45.1 ± 5.5** (b)(c)(e)(f)	48.7 ± 3.9 (b)(d)(e)(f)	**38.7 ± 2.3** (b)(f)	**36.6 ± 2.1** (b)(e)(f)
Glu-Gly	40.1 ± 1.1 (b)	44.0 ± 4.6 (b)(f)	41.9 ± 5.0 (a)(b)(f)	39.1 ± 3.1 (b)(f)	44.4 ± 4.3 (b)(f)	**36.3 ± 4.3** (a)(b)(f)	45.8 ± 3.5 (b)(d)(e)(f)	**36.6 ± 2.2** (b)(f)	32.1 ± 2.9 (b)(f)
ChCl-Glu	36.5 ± 3.1	41.9 ± 4.9 (b)(f)	46.9 ± 3.7 (b)(f)	**37.1 ± 3.2** (a)(b)(f)	**39.4 ± 3.3** (a)(b)(f)	**38.5 ± 3.1** (a)(b)(c)(f)	**33.3 ± 3.0** (a)(b)(c)(f)	**33.4 ± 2.8** (a)(b)(f)	**29.9 ± 3.1** (a)(b)(d)(f)
ChCl-CitAc	36.4 ± 3.8	43.3 ± 3.6 (b)(e)(f)	44.1 ± 7.4 (b)(f)	**34.8 ± 8.7** (a)(f)	40.9 ± 6.4 (b)(f)	**35.6 ± 3.5** (a)(b)(f)	**41.7 ± 1.5** (a)(b)(c)(f)	**34.2 ± 1.8** (a)(b)(f)	**35.9 ± 1.6** (b)(e)(f)
Ethanol-Water	32.9 ± 1.6	27.2 ± 2.3 (a)(c)(d)	32.6 ± 3.2 (a)(c)(d)(e)	**25.0 ± 1.4** (a)(c)(d)(e)	**26.2 ± 1.4** (a)(c)(d)(e)	**24.8 ± 0.9** (a)(c)(d)(e)	**28.0 ± 1.2** (a)(b)(c)(d)(e)	**17.4 ± 1.1** (a)(c)(d)(e)	**14.8 ± 3.7** (a)(b)(c)(d)(e)
CitAc-Glu	26.9 ± 9.8 (a)(c)	27.7 ± 8.1 (a)(b)(c)(d)	24.8 ± 7.1 (a)(c)(d)(e)	27.0 ± 7.1 (a)(c)(e)	29.2 ± 8.6 (a)(c)(d)(e)	**22.2 ± 2.5** (a)(c)(d)(e)	**14.8 ± 1.0** (a)(c)(d)(e)(f)	**21.6 ± 4.1** (a)(c)(d)(e)	**22.0 ± 3.4** (a)(c)(d)(e)(f)

In bold: significant differences with p-value < 0.001 within the same NADES with respect to the day. [(a)] Significant differences < 0.001 with respect to ChCl-Gly; [(b)] significant differences < 0.001 with respect to CitAc-Glu; and [(c)] significant differences < 0.001 with respect to Glu-Gly. [(d)] Significant differences < 0.001 with respect to ChCl-CitAc. [(e)] Significant differences < 0.001 with respect to ChCl-Glu and [(f)] significant differences < 0.001 with respect to ethanol–water.

The best results were achieved with NADES extracts from the two combinations with Gly, without presenting significant differences. The best inhibition and its evolution was obtained from the ChCl-Gly extracts at 52%; followed by the Glu-Gly extracts with a maximum inhibition of 45.8%. The combinations of ChCl with Glu or with CitAc were good results too, finding 46.9% and 44.1% reduction in DPPH, respectively. Both showed significant differences from the Gly combinations as the trial progressed. The worst results, which were more variable and showed significant differences with respect to previous ones, were the extracts from CitAc-Glu, with up to a 29% reduction in DPPH. It should be noted that this last extract was the only one that did not exceed the values obtained with a conventional reference solvent (ethanol–water), although they were statistically similar. On the other hand, from this reference solvent, a significant difference with respect to the first four named solvents was observed.

Table 3 shows that the highest retention or stability of TAC was shown by the ChCl-CitAc extract at 98.3%, while the greatest reduction was seen in the ethanol–water extract at 43.5%, which behaved differently from the other extracts that presented withholdings of

around 80–90%. Moreover, Table 3 shows how the stability of TAC was maintained until day 15 of analysis. From that day, the values of all extracts decreased.

Table 3. Total antioxidant capacity retention during the stability test.

Solvents	TAC Day 1 (% Inhibition)	TAC Day 15 (% Inhibition)	TAC Day 24 (% Inhibition)	Retention Day 15 (%)	Retention Day 24 (%)
ChCl-Gly	41.7	45.1	36.6	100	87.5
Glu-Gly	40.1	36.3	32.1	95.5	82.2
ChCl-Glu	36.5	38.5	29.9	100	81.4
ChCl-CitAc	36.4	35.6	35.9	97.3	98.3
Ethanol–water	32.9	24.8	14.8	74.5	43.5
CitAc-Glu	26.9	22.2	22.0	77.9	77.5

TAC, total antioxidant capacity.

3.2. Measurement of the Content of Total Polyphenolic Compounds

The estimation of TPC was represented in mg galic acid equivalent (GAE)/100 g dry weight (DW) orange peel. The changes were observed during the stability test (Table 4).

Table 4. Comparison of total polyphenolic compounds for different tested NADES.

mg GAE/ 100 g DW	Day 1	Day 2	Day 3	Day 8	Day 10	Day 15	Day 17	Day 22	Day 24
CitAc-Glu	5060 ± 60 (a)(b)(d)(e)(f)	5090 ± 110 (a)(b)(d)(e)(f)	5180 ± 50 (a)(b)(d)(e)(f)	5060 ± 60 (a)(b)(d)(e)(f)	4800 ± 110 (a)(b)(d)(e)(f)	**4620 ± 90** (a)(b)(d)(e)(f)	**4340 ± 30** (a)(b)(d)(e)(f)	**4410 ± 70** (a)(b)(d)(e)(f)	**4420 ± 80** (a)(b)(d)(e)(f)
Ethanol-Water	4680 ± 180 (a)(b)(c)(d)(e)	4470 ± 130 (a)(b)(c)(d)(e)	4320 ± 30 (a)(b)(c)(d)	**3700 ± 230** (a)(b)(c)(d)(e)	**3820 ± 150** (a)(b)(c)(d)	**4010 ± 110** (a)(b)(c)(d)(e)	**3760 ± 100** (a)(b)(c)(d)(e)	**3900 ± 80** (a)(b)(c)(d)(e)	**3730 ± 70** (a)(b)(c)(d)(e)
Glu-Gly	4050 ± 70 (a)(b)(c)(d)(e)(f)	3930 ± 100 (a)(b)(c)(d)(e)	4670 ± 90 (a)(b)(c)(d)(e)	**3970 ± 70** (a)(b)(c)(d)(f)	**4010 ± 120** (a)(b)(c)(d)(f)	**3620 ± 130** (a)(b)(c)(d)(f)	**3480 ± 140** (a)(b)(c)(d)(f)	**3220 ± 220** (a)(b)(c)(d)(f)	**3390 ± 120** (a)(b)(c)(d)(f)
ChCl-CitAc	1380 ± 70 (a)(c)(d)(e)(f)	1230 ± 50 (a)(c)(e)(f)	1310 ± 50 (a)(c)(e)(f)	1500 ± 80 (a)(c)(d)(e)(f)	1540 ± 40 (a)(c)(d)(e)(f)	**800 ± 80** (c)(e)(f)	**1020 ± 30** (a)(b)(c)(e)(f)	**1180 ± 40** (a)(c)(d)(e)(f)	**1220 ± 40** (a)(c)(d)(e)(f)
ChCl-Glu	950 ± 30 (b)(c)(e)(f)	1000 ± 30 (c)(e)(f)	1220 ± 30 (a)(c)(e)(f)	920 ± 30 (b)(c)(e)(f)	1180 ± 20 (b)(c)(e)(f)	**880 ± 50** (c)(e)(f)	**830 ± 20** (c)(e)(f)	**810 ± 30** (b)(c)(e)(f)	**810 ± 20** (b)(c)(e)(f)
ChCl-Gly	740 ± 30 (b)(c)(e)(f)	840 ± 30 (b)(c)(e)(f)	790 ± 30 (b)(c)(d)(e)(f)	820 ± 30 (b)(c)(e)(f)	1060 ± 30 (b)(c)(e)(f)	740 ± 10 (c)(e)(f)	**590 ± 20** (b)(c)(e)(f)	**650 ± 30** (b)(c)(e)(f)	**700 ± 30** (b)(c)(e)(f)

In bold: Significant differences with p-value < 0.001 within the same NADES with respect to the day. [(a)] Significant differences < 0.001 with respect to ChCl-Gly; [(b)] significant differences < 0.001 with respect to ChCl-CitAc; and [(c)] Significant differences < 0.001 with respect to CitAc-Glu. [(d)] Significant differences < 0.001 with respect to ChCl-Glu. [(e)] Significant differences < 0.001 with respect to Glu-Gly and [(f)] significant differences < 0.001 with respect to ethanol–water. GAE, galic acid equivalent. DW, dry weight.

In this case, data are different from those seen in the TAC measurements. There are two clearly differentiated groups with significant differences among them, with the worst results being for the three NADES extracts that present ChCl in their combinations. The results of the CitAc-Glu extract stand out, seeing that it showed the best results in terms of TPC extraction with a maximum of 5180 mg GAE/100 g DW and presenting significant differences with respect to all other extracts. The values of the mixtures with ethanol–water and Glu-Gly followed, which reached a concentration of up to 4680 and 4670 mg GAE/100 g DW, respectively. The three remaining mixtures with ChCl reached a maximum concentration of between 1540 (ChCl-CitAc) and 590 mg GAE/100 g DW (ChCl-Gly), which was very significant if we compare the value with 3220 mg GAE/100 g of DW, which was the lowest value for the Glu-Gly extract.

In Table 5, the highest retention of TPC at the end of the test was demonstrated by the ChCl-Gly extract at 94.6%, while the greatest reduction was shown by the ethanol–water at 79.7%. All other extracts had TPC levels of around 85%. In this test, NADES continued to

show better retention results than those obtained using a conventional solvent. As with Table 3, the stability of TPC was maintained until day 15 of analysis.

Table 5. Total polyphenolic compounds retention during the test.

Solvents	TPC Day 1	TPC Day 15	TPC Day 24	Retention Day 15 (%)	Retention Day 24 (%)
CitAc-Glu	5060	4620	4420	91.3	87.4
Ethanol–water	4680	4010	3730	85.7	79.7
Glu-Gly	4050	3620	3390	89.4	83.7
ChCl-CitAc	1350	800	1220	59.3	90.4
ChCl-Glu	950	880	810	92.6	85.3
ChCl-Gly	740	740	700	100	94.6

TPC, total polyphenolic compounds (mg GAE/100 g DW).

4. Discussion

In this section, the relevance of the use of more hydrophilic NADES for the extraction of polar compounds is discussed. The results obtained are compared with other studies using NADES to show that they are a significant alternative in the extraction of polar bioactive compounds, such as TPC, and acquire a better TAC.

Two of the selected studies that used citrus peel (orange and lemon) have been included. In the first study, the performance of NADES at 30, 50, or 80% water (*v*:*v*) to extract TPC from orange peel was analyzed [3] and the best results were found to be the combinations of NADES 50 and 80% water (5200 and 5100 mg GAE/100 g fresh weight (FW), respectively). The ethanol reference extract obtained 4000 mg GAE/100 g FW, thus, a better performance was observed for the more hydrophilic NADES extracts. In contrast, the other study (using lemon peel) [9] verified the TPC content of both conventional extracts (60% ethanol, *v*:*v*) and NADES tested in 10% water. In this case, the performance of TPC content with conventional extract was better than NADES extract, with 5370 compared to the 2710 mg GAE/100 g DW, respectively. However, when comparing with the previous study, it can be observed that there is a better performance if values express the results in terms of dry weight. Both studies showed results similar to those obtained in this work.

Among the studies with the best results is [12], which shows a process for extracting TPC from coffee using NADES at 50% water combined with US. The highest TPC content was 8701 mg GAE/100 g DW of extracted coffee. Therefore, a better performance was also observed here for the more hydrophilic NADES extracts.

Regarding TAC, studies found better results with less hydrophilic NADES extracts (20% water), between 92 and 73% inhibition of DPPH [3,13]. While an extract of NADES in 70% water = achieved a 59% reduction in DPPH [14], which was similar to the 52% obtained in this work.

Finally, regarding the stability of the tests, it should be noted that no articles were found that use extracts of NADES in this type of evaluation, so comparisons were carried out using studies that used conventional solvents. The retention of TPC in these studies was between 93–99% [15] and 66–85% [16], while that in this work was 83.7–94.6% for NADES and 79.7% (ethanol–water) and are within the same range. However, in terms of TAC, studies obtained a 97–99% [15] and 70–82% [16,17] retention, while that in this work was 77.5–98.3% in NADES and 43.5% (ethanol–water). This may indicate that the ethanol–water extract obtained other antioxidant compounds, apart from polyphenols, such as vitamin C, which is more sensitive to light and temperature and, therefore, thus resulting in the difference in final TAC. In the other extracts, there seemed to be a correlation between the reduction in TPC and TAC in the NADES, from day 15 at 4 °C.

5. Conclusions

1. The highest TPC extraction was obtained with NADES CitAc-Glu with 5180 mg GAE/100 g DW, with significant differences compared to the other extracts and the ethanol–water extract (50%, *v*:*v*) with 4680 mg GAE/100 g dry weight.
2. The highest TAC was obtained with the NADES (ChCl-Gly and Glu-Gly) with up to a 52 and 45.8% reduction in DPPH, but ethanol–water (50%, *v*:*v*) achieved a 29.2% reduction.
3. The retention of TPC in 4 weeks of analysis was between 83.7–94.6% for the NADES, while that of ethanol–water (50%, *v*:*v*) decreased to 79.7%.
4. TAC retention in 4 weeks of analysis was between 77.5–98.3% in the NADES while that of ethanol–water (50%, *v*:*v*) was the lowest with 43.5%.
5. It was found that a higher proportion of water in NADES led to better TPC and TAC extractions compared to most studies that used smaller amounts.
6. The stability of TPC and TAC was maintained until day 15 of analysis, showing a correlation in the reduction of TPC with TAC, except for the ethanol–water extract, where other antioxidants were able to intervene.

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