Foods 2021

MDPI

Proceedings

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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† Presented at the 2nd International Electronic Conference on Foods, 15–30 October 2021; Available online: https://foods2021.sciforum.net/.

Abstract: The reuse of food chain residues is topical. This revaluation can extract bioactive compounds of 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 very low environmental impact. The tests were performed with 5 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 obtaining significant changes from day 15 of storage at 4 °C.

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

Citation: Tejero-Martínez, A., Martín, M.E., Malo-López, D., Frígola, A., Esteve M.J., Blesa, J..; Assessment of the use of a selection of natural deep eutectic solvents in the extraction of polar bioactive compounds from orange peel. *Proceedings* **2021**, *68*, x.

Published: 15 October 2021

1. Introduction

In recent years the concern for preserving the environment has become increasingly important. Food industry is trying to find ways to reduce its carbon footprint and pollution of theirs processes, make better use of food and reuse its residues. By the way, 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 need of 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 is one of the most abundant in the food industry. 70 million tons of oranges are produced annually worldwide, among which the peel comprises 40-50% of the total weight of the orange [3].

During the last twenty years, there have been two focuses of study regarding "green solvents". One of them has been on 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 the research has been received by the other focus of

study, the Deep Eutectic Solvents (DES) [4]. They are gaining more importance due to the investigations that are carried out year after year.

The definition given for DES [4] consist of liquid mixture, formed by two or more compounds, whose melting point 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 DES formed by natural compounds of low molecular weight as sugars, organic acids, or amino acids among others.

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

2. Materials and Methods

2.1. Sample Preparation and Extraction Procedure.

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

2.2. NADES Preparation.

The choice of the mixtures was made through a previous bibliographic search, opting for those where better results were obtained on 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.

It was used various possibilities to create NADES (quaternary amine, sugar, organic acid, and glycerol). Their components were choline chloride (ChCl), glucose (Glu), citric

acid (CitAc) and glycerol (Gly), using the same stoichiometric proportion of the original studies. Subsequently, they were heated to between 50 and 90 °C in a water bath to facilitate their melting and then, the 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.

Measurement of TAC was using the DPPH method [10]; meanwhile the measurement of the content of TPC were quantified using the Folin-Ciocalteau method [11].

2.4. Statistical analysis

A 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 by the ANOVA test. A post hoc test of multiple comparisons, HSD Tukey, was used to check for differences between the extracts per day.

3. Results.

3.1. Total Antioxidant Capacity.

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

Table 2. Comparison of Total Antioxidant Capacity for different NADES tested.

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-AC	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)
Etanol-Agua	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)
AC-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 AC-Glu; (c) Significant differences <0.001 with respect to Glu-Gly. (d) Significant differences <0.001 with respect to ChCl-AC. (e) Significant differences <0.001 with respect to ChCl-Glu; (f) Significant differences <0.001 with respect to Ethanol-Water.

The best results have been achieved in the NADES extracts from the two combinations with Gly without presenting significant differences. The best inhibition was obtained by ChCl-Gly extracts with 52%; followed by Glu-Gly extracts with a maximum inhibition of 45.8%. The following combinations with the best results were ChCl with Glu or with CitAc, finding between 46.9 and 44.1% reduction in DPPH, respectively. Both showed significant differences with the Gly combinations as the trial progressed. The worst results, more variable and with significant differences with respect to the previous ones, have been the extracts from CitAc-Glu with up to 29% reduction in DPPH. It should be noted that this last extract is the only one that does not exceed the values obtained with a conventional reference solvent (Ethanol-Water), although they are statistically similar. On the other hand, from this reference solvent it is observed that there is a significant difference with respect to the first 4 named solvents.

Table 3 shows that the highest retention or stability of TAC was shown by the ChCl-CitAc extract with 98.3%, while the greatest reduction was seen in the Ethanol-Water extract with 43.5% behaving differently from the other extracts that presented withholdings around 80-90%. Moreover, Table 3 shows how the stability of TAC has been maintained until day 15 of analysis. Since this day the values of all extracts has been 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
Etanol-Water	32.9	24.8	14.8	74.5	43.5
CitAc-Glu	26.9	22.2	22.0	77.9	77.5

3.2. Measurement of the content of Total Phenolic Compounds.

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

Table 4. Comparison of Total Phenolic Compounds for different NADES tested.

mg AG/100g 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; (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; (f) Significant differences <0.001 with respect to Ethanol-Water.

In this case, data is different from those seen in the TAC measurement. There are two clearly differentiated groups with differences significant among them, with the worst results being the three NADES extracts that present ChCl in their combinations. The results of the CitAc-Glu extract stand out, seeing it as the best in terms of TPC extraction with a maximum of 5180 mg GAE / 100 g DW and presenting significant differences with respect to all the others. 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 reach a maximum concentration of between 1540 (ChCl-CitAc) and 590 mg GAE / 100 g DW (ChCl-Gly), which is very significant if we compare it with 3220 mg GAE / 100 g of DW, which is the lowest value of Glu-Gly extract.

In Table 5 the highest retention of TPC at the end of the test was shown by the ChCl-Gly extract with 94.6%, while the highest reduction was presented by the Ethanol-Water

with 79.7 %. All other extracts kept TPC levels around 85%. In this test the NADES continue to show better retention results than those obtained by a conventional solvent. In the same way that happen in Table 3; the stability of TPC is maintained until day 15 of analysis.

Table 5. Total Polyphenolic Compounds retention during the test.

Solvents	TPC day 1 (mg GAE/100 g DW)	TPC day 15 (mg GAE/100 g DW)	TPC day 24 (mg GAE/100 g DW)	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

4. Discussion.

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

Two of the selected studies that used citrus peel (orange and lemon) have been included. In the first it analyzed the performance of NADES at 30, 50, or 80% water (v:v) to extract TPC from orange peel [3] and found that the best results were those of the combinations of NADES 50 and 80% water (5200 and 5100 mg GAE/ 100 g fresh weight (FW), respectively). While the ethanol reference extract obtained 4000 mg GAE / 100 g FW, thus, better performance is 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 to extract TPC was better for the NADES extract with 5370 compared to the 2710 mg GAE / 100 g DW achieved by the ethanol extract. However, when comparing with the previous study, it would be observed a better performance if have been expressed the results in dry weight. Both studies have shown results within the range obtained by this work.

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

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

Finally, regarding the stability of the tests, it should be noted that now have not been found articles that use extracts of NADES in this evaluation, so the comparisons have been carried out with studies that used conventional solvents. The retention of TPC in the studies was between 99-93% [15] and 85-66% [16] while that of this work has been 94.6-83.7% in NADES and 79.7% (Ethanol -Water), so they are within the same range. However, in terms of TAC, the studies obtained a 99-97% [15] and 82-70% [16,17] retention, while that of this work has been 98.3-77.5% in NADES and 43.5% (Ethanol-Water). This may indicate that the Ethanol-Water extract has obtained other antioxidant compounds apart from polyphenols such as vitamin C which is more sensitive to light or temperature and therefore there has been this difference in final TAC. In the other extracts there seems to be a correlation between the reduction of TPC and TAC in the NADES from day 15 at 4 °C.

5. Conclusions.

1. The highest TPC extraction was obtained by NADES AC-Glu with 5180 mg GAE / 100g DW with significant differences compared to the rest and the Ethanol-Water extract (50%, v:v) with 4680 mg GAE / 100 g weight dried.

2. The highest TAC was obtained by the NADES (ChCl-Gly and Glu-Gly) with up to 52 and 45.8% reduction in DPPH but Ethanol-Water (50%, v: v) achieved 29.2% reduction.

3. The retention of TPC in 4 weeks of analysis has been between 94.6 and 83.7% in the NADES while that of the Ethanol-Water (50%, v:v) decreased to 79.7%.

4. TAC retention in 4 weeks of analysis has been between 98.3 and 77.5% in the NADES while that of Ethanol-Water (50%, v:v) was the lowest with 43.5%.

5. It has been found that a higher proportion of water in NADES has led to better TPF and TAC extractions than most studies using smaller amounts.

6. The stability of TPC and TAC has been maintained until day 15 of analysis, showing correlation in the reduction of TPC with TAC except for the Ethanol-Water extract where other antioxidants have been able to intervene.

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