IFSCC 2025 full paper (IFSCC2025-713)

From Waste to Value: Green Extraction of Phenolics from Orange Peels with NADES

Marta Marques[1,2]*, Ana Rita Jesus[1], Ana Rita Duarte[1,2], Alexandre Paiva[1,2]

[1] LAQV-REQUIMTE, Departamento de Química, Nova School of Science and Technology, 2829-516 Caparica, Portugal;
[2] DES Solutio - Scientific Consulting Solutions Lda, Rua dos Inventores, 2825-185 Caparica, Portugal
*Corresponding author email: ma.marques@campus.fct.unl.pt

1. Introduction

Waste reduction has become a global priority in recent years, driven by the increase in environmental and economic concerns. Among the industries that generate the most waste, the agricultural and food industries stand out due to the large quantities of by-products produced during harvesting and processing. For instance, peels, pomace, and seeds are often discarted despite their hidden value. Both vegetable and fruit by-products are rich sources of bioactive compounds such as vitamins, proteins, flavonoids, carotenoids, polyphenols, anthocyanins and pigments. These compounds exhibit various therapeutic properties including antioxidant, anti-inflammatory and anticancer activities [1]–[3], and can be applied in nutraceuticals, cosmetics, and functional foods.

As reported by *Ben-Othman et al. (2020)*, polyphenolic content is approximatly 15% higher in the peels of apple, grapes, citrus fruits, as well as in the seeds of jackfruit, avocado and mango compared to their respective pulps [4]. According to data from the Food and Agriculture Organization (FAO), oranges were among the five most produced fruits globally in 2023, with an estimated production of approximately 70 million tonnes [5]. In particular, oranges are widely processed for juice production, with approximately 50% of orange fruits being used for this purpose, generating substantial amounts of by-products such as peels, seeds, and pulp [6]–[8]. A significant portion of these residues, mainly orange peels, which represent approximately 50% of the fruit, are often discarded as waste following juice production [9].

Orange peels are rich in valuable components such as essential oils, soluble sugars, pectin, cellulose, hemicellulose, and other bioactive compounds, including phenolic compounds. The main phenolic compounds found in orange peels are flavonoids, such as hesperidin, naringin, rutin, and quercetin, as well as hydroxycinnamic acids like ferulic acid and caffeic acid [6], [9] [7]. The main processes used for orange peel valorisation focus on the recovery of essential oils and pectin. Recently, there has been increasing interest in recovering phenolic compounds. Conventional methods for extracting phenolic compounds from citrus waste include solvent

extraction, hot water extraction, alkaline extraction, resin-based extraction, enzyme-assisted extraction, and supercritical fluid extraction [7]. However, most of the solvents used for extraction are organic solvents, which bring environmental concerns due to their toxicity and high environmental impact. Consequently, there is an increasing need for more sustainable extraction methods. In this context, deep eutectic systems (DES) have emerged as a promising, eco-friendly alternative, offering a more sustainable approach to the extraction of bioactive compounds from orange peels.

Deep eutectic systems (DES) were first introduced by Abbott et al. (2003) as mixtures of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), typically solid at room temperature, which form a liquid when combined in specific molar ratios [10]. The interaction between the hydrogen bond donor and acceptor, is responsible for the lower melting point of the eutectic mixture compared to the raw materials [11]–[13]. The preparation of DESs is very straightforward, requiring only the mixing and heating of the components without any chemical reaction, thereby achieving a 100% atom economy process [12]. They exhibit low toxicity, non-flammability, low volatility, and good chemical and thermal stability [14], [15]. Moreover, their properties can be tuned by varying the nature of the HBD and HBA [15], [16]. When DESs are composed of naturally occurring metabolites, they are referred to as natural deep eutectic systems (NADES). These metabolites typically include sugars, sugar alcohols, amino acids, and organic acids, often with water incorporated as a third component [11], [17], [18].

The main objectives of this work were to: (a) evaluate the potential of three NADES on the extraction of bioactive compounds. Extracts were evaluated for their total phenolic content and antioxidant activity; (b) optimize extraction conditions – time, temperature and ratio orange peels:NADES; (c) evaluate the cytotoxicity and the ability to inhibit the formation of reactive oxygen species (ROS) in an immortalized cell line.

2. Materials and Methods

2.1. Materials

2.1.1. Plant material

Orange peels (OP) used in this study were obtained from fresh oranges consumed in a domestic setting. Prior to processing, orange peels were lyophilized and ground (IKA tube-Mill control, Germany) to obtain particles with sizes under 0.5 mm (1 min at 6000 rpm).

2.1.2. Chemicals

Sucrose (≥99.5%), D-(+)-glucose (≥99.5%), DL-lactic acid (85.0%), gallic acid monohydrate (≥98.0%), Folin-Ciocalteu's phenol reagent 2N and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were acquired from Sigma-Aldrich (St. Louis, Missouri, USA). Betaine anhydrous (≥97.0%) was obtained from TCI-Tokyo Chemical Industry (Tokyo, Japan), glycerol (≥99.0%) from Honeywell (Charlotte, North Carolina, USA), ethanol (96.0%) from LabChem (Zelienople, Pennsylvania, USA) and methanol (≥99.9%) from Carlo Erba (Milan, Italy).

2.2. Methods

2.2.1. Preparation of NADES

The method utilized for the preparation of the Natural Deep Eutectic Solvents was the heating and stirring method previously reported by Dai and co-workers [17]. First, the individual compounds were carefully weighed and then combined, according to the defined molar ratios, and stirred in a water bath at approximately 50 °C until a transparent liquid was obtained. The Natural deep eutectic systems used in this study will be addressed as NADES1, NADES2 and NADES3, and their respective extracts as ExtNADES1, EXTNADES2 and ExtNADES3.

2.2.2. Solid-Liquid extraction of bioactive compounds from orange peels powder

The solid-liquid extractions using either the conventional solvent and NADES, were performed with slight modifications, following the methods of Ozturk and co-workers and El Kantar and co-workers [19], [20].

For the conventional extraction using heating and stirring method, orange peels powder was mixed with a hydroalcoholic solution of 50:50 (v/v) EtOH:H_2O in a solid/liquid ratio (S/L) of 1:10. The extraction was performed at 40 °C in a water bath for 60 minutes. The obtained extract was centrifuged (6000 rpm and 25 minutes, Hermle) and the supernatant was stored at 4 °C until further use. The extraction was performed in triplicate.

For the NADES-based heating/stirring extraction, the orange peels powder was mixed with NADES at an optimized S/L (g/mL) of 1:10. The extractions were performed at 40 °C (water bath) for 60 minutes. The obtained extract was then centrifuged at 6000 RPM for 20 minutes (Hermle). The supernatant was recovered and stored at 4 °C.

2.2.3. Determination of the total phenolic content

The colorimetric Folin-Ciocalteu method described by Singleton and Rossi in 1965 was used to determine the total phenolic content (TPC) of the extracts [21]. A calibration curve was prepared using gallic acid as a standard. For the assay, 20 µL of each sample was mixed with 1.58 mL of distilled water and 100 µL of Folin-Ciocalteu reagent. The mixture was incubated at room temperature for 5 minutes. Then, 300 µL of a saturated sodium carbonate (Na_2CO_3) solution was added, and the mixture was stirred. The samples were incubated at 40 °C for 30 minutes in a dry bath (Labnet D1200). After incubation, absorbances were measured at 750 nm using a UV spectrophotometer (Thermo Scientific, Genesys 50). The total phenolic content was calculated and expressed as mg of gallic acid equivalents per litre (mg GAE/L).

2.2.4. Determination of the antioxidant activity

The antioxidant activity of the extracts was determined using the colorimetric DPPH assay [22]. A stock solution was prepared by dissolving 24 mg of DPPH (2,2-diphenyl-1-picrylhydrazyl) in 100 mL of methanol and stored at -20 °C for at least 2 hours. The working solution was prepared by diluting 10 mL of the stock solution in 45 mL of methanol. The absorbance of the working solution was measured at 517 nm using a UV spectrophotometer (Thermo Scientific, Genesys 50). Afterwards, 150 µL of different concentrations of the extract was mixed with 4 mL of the DPPH working solution. For the blank solution, 150 µL of distilled water was added to 4 mL of the working solution. All mixtures were stirred, incubated in the dark at room temperature for 40 minutes and absorbances measured at 517 nm.

To determine the radical scavenging activity (% RSA) the following equation was used:

$$\% \, RSA = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100$$

where A_{sample} is the absorbance of the sample and $A_{control}$ is the absorbance of the blank. The % RSA indicates the percentage of DPPH radicals scavenged by the antioxidant.
The EC50 (half maximum effective concentration), which is the concentration of an antioxidant that scavenges 50% of DPPH radicals, was obtained by plotting extract concentration against % RSA.

2.2.5. Biological Evaluation

2.2.5.1. Cytotoxicity in L-929 cell line

The cytotoxicity of NADES-based extracts were evaluated using the L-929 cell line (DSMZ-German Collection of Microorganisms and Cell Cultures GmbH), are a fibroblast-like cell line derived from the subcutaneous connective tissue of a 100-day-old male C3H/An mouse.
L-929 cells were incubated in a 96-well plate at a density of 1×10^5 cells/mL until confluency was reached (24 h). Afterwards, the NADES-based extracts were added at concentrations ranging from 6.25 to 200 mg/mL and incubated for 24 h at 37 ºC and 5% CO_2. In the case of ExtNADES1 and ExtNADES2, samples were prepared by directly dissolving the samples in culture media.
Control cells were incubated with complete media. To evaluate cell viability, CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega), based on MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetra-zolium), was carried out. The amount of formazan product was measured in a microplate reader (VICTOR Nivo TM, PerkinElmer, USA) at 490 nm, as absorbance is directly proportional to the number of viable cells in culture. Cell viability was expressed as percentage of cells exposed to extracts vs control.

2.2.5.2 In vitro ROS inhibition - Induced by H_2O_2

To determine the ability of NADES-based extracts to inhibit the formation of ROS, L-929 cells were incubated with the NADES-based extracts and ROS formation was stimulated using H_2O_2.
L-929 cells were incubated in a 96-well plate at a density of 1×10^5 cells/mL until confluency was reached (24 h). L-929 cells were incubated for 30 minutes with 20 µM 2,7'dichlorodihydrofluorescein diacetate (H2-DCFDA, Sigma) in the dark at 37 ºC with 5% CO_2. After removal of probe solution, ExtNADES1 was added at concentrations ranging from 6.25 to 100 mg/mL and ExtNADES2 was added at 6.25-50 mg/mL. Ascorbic acid (1% w/v) was used as positive control. After 1 h incubation, without removing the media, hydrogen peroxide (H_2O_2) was added to a final concentration of 500 µM. ROS levels were determined at 485 nm (excitation) and 520 nm (emission) wavelengths using a fluorescence microplate reader.
The % of ROS inhibition was determined using the following equation:

$$\%\text{ROS inhibition} = \left(1 - \frac{FI_{sample}}{FI_{H2O2}}\right) \times 100$$

where FI_{sample} is the fluorescence intensity of each sample and FI_{H2O2} is the fluorescence intensity of cells incubated only with H_2O_2.

3. Results

In this study, three NADES were evaluated for their efficiency in extracting bioactive compounds from dry orange peels. Total phenolic content and antioxidant activity were first compared to a conventional extraction. Two NADES were then optimized to improve the

effenciency of the extraction, and biological activity (cytotoxicity and ROS inhibition) of the extracts was also assessed using L-929 cell line.

3.1. Conventional and NADES-based extraction

The obtained extracts were characterized for their total phenolic content (TPC) and antioxidant activity (EC_{50}), providing a comprehensive evaluation of each solvent's performance. The total phenolic content and antioxidant activity of the obtained extracts are presented in Table 1.

Table 1 - Total phenolic content (mg gallic acid equivalent/g dry weight orange peels) for the obtained NADES extracts and conventional extract (50:50 v/v Ethanol:H_2O). Data represents mean ± SD (n = 3). Antioxidant activity (measured as EC_{50}, g extract/mL) for the obtained NADES extracts and conventional extract (50:50 v/v Ethanol:H_2O).

Orange peels extracts	TPC (mg GAE/ g dry OP)	EC_{50} (g extract/ mL)
ExtConventional	21.37 ± 0.64	1.10
ExtNADES 1	12.60 ± 0.55	0.61
ExtNADES 2	15.12 ± 0.85	0.78
ExtNADES 3	16.78 ± 0.06	*

*not able to determine the EC_{50} - %RSA was lower that 50%

As shown in Table 1, the extract that exhibited the highest total phenolic content (TPC) compared to the conventional extract (ExtConventional) was ExtNADES3, with a value of 16.78 ± 0.06 mg GAE/g dry OP, followed by ExtNADES2 with 15.12 ± 0.85 mg GAE/g dry OP. On the other hand, ExtNADES1 (12.60 ± 0.55 mg GAE/g dry OP) presented significantly lower TPC values, indicating a limited efficiency in recovering phenolic compounds when compared to both the conventional method and the other NADES extracts. Regarding antioxidant activity, measured as EC_{50}, the results followed a different trend. For ExtNADES3, it was not possible to determine the EC_{50}, as the %RSA remained below 50%. In contrast, both ExtNADES1 (0.61 g extract/mL) and ExtNADES2 (0.78 g extract/mL) demonstrated better antioxidant activity than the conventional extract (1.10 g extract/mL).

3.2. Optimization of extraction conditions for ExtNADES1 and ExtNADES2

The extraction conditions for ExtNADES1 and ExtNADES2 were optimized by evaluating the effects of time, solid-to-liquid ratio (S/L), and temperature on the total phenolic content (TPC).

When using ExtNADES1, total phenolic content increased over time, reaching a maximum at 3 hours, with a 66.9% higher extraction yield compared to 30 minutes. Longer extraction times did not significantly improve the yield and may even result in a slight decrease, suggesting possible degradation of phenolic compounds.

Regarding the solid-to-solvent ratio, a 1:20 ratio yielded slightly higher TPC values compared to 1:10, with only a 13.6% increase. Increasing the extraction temperature also enhanced TPC, with the highest values observed at 40 °C and 50 °C, representing increases of 28.6% and 42.5%, respectively, when compared to 30 °C.

These findings suggest that the optimal conditions for extracting phenolic compounds from orange peels using NADES1 are 3 hours of extraction, a solid-to-solvent ratio of 1:10 or 1:20, and a temperature of 50 °C.

The extraction of phenolic compounds using NADES2 was also influenced by time, solid-to-liquid ratio, and temperature. Regarding extraction time, a 5-hour extraction led to a 47.9% increase in total phenolic content (TPC) compared to 30 minutes, and a 22.0% increase compared to 1 hour. However, the increase in total phenolic content from 3 to 5 hours was only 10.2%, indicating that the majority of phenolic compounds were extracted within the first 3 hours of the process. For the solid-to-liquid ratio, increasing the ratio from 1:10 to 1:20 resulted in a 24.1% increase in TPC, indicating improved mass transfer at higher solvent volumes. Temperature also had a significant impact: extraction at 50 °C led to increases of 25.2% and 16.2% in TPC when compared to extractions at 30 °C and 40 °C, respectively. Overall, the optimal conditions for phenolic compound extraction using NADES2 were identified as 3 hours of extraction, a solid-to-liquid ratio of 1:20, and a temperature of 50 °C.

3.3. Biological evaluation

The biological evaluation was assessed using the immortalized L-929 cell line, which is derived from mouse connective tissue. For this evaluation, the obtained ExtNADES1 and ExtNADES2 were used. The cytotoxicity of the extracts was assessed to determine their safety profile.

ExtNADES1 showed no cytotoxic effects up to a concentration of 100 mg/mL, maintaining cell viability above 80%. In contrast, ExtNADES2 exhibited cytotoxicity at 100 mg/mL, with a cell viability of approximately 70%. According to *ISO 10993-5*, a cell viability below 70% is considered indicative of cytotoxicity, providing a benchmark for biocompatibility assessment. Therefore, ExtNADES1 was considered non-cytotoxic at concentrations up to 100 mg/mL, while ExtNADES2 was safe only at concentrations up to 50 mg/mL.

The ability to inhibit the formation of reactive oxygen species (ROS) induced by hydrogen peroxide was also evaluated. Additionally, a cell viability assay was performed 24 hours after exposure to ExtNADES1 and ExtNADES2, to assess the cytotoxicity of the extracts following ROS inhibition.

The evaluation of ROS inhibition demonstrated that both ExtNADES1 and ExtNADES2 were highly effective in preventing the formation of reactive oxygen species; across all tested concentrations (6.25–100 mg/mL), the extracts maintained a strong antioxidant capacity, comparable to that observed with 10 mg/mL ascorbic acid, which used as the positive control. Regarding cytotoxicity, cell viability assays performed 24 hours post-treatment indicated that exposure to either extract preserved cell viability above 70% at all concentrations tested. In contrast, cells treated with 10 mg/mL ascorbic acid exhibited notably lower viability.

4. Discussion

Recent studies have explored the extraction of phenolic compounds from orange peels using NADES, with a particular focus on choline chloride-based systems. For example, Ozturk and co-workers demonstrated that choline chloride:ethylene glycol (1:4) exhibited greater selectivity and antioxidant activity than hydro-ethanolic solution (2.62 mg GAE/g OP), achieving a TPC

of 3.61 mg GAE/g OP under optimized conditions [19]. In another study, Gómez-Urios et al. (2022) evaluated various NADES for the extraction of bioactive compounds, where lactic acid:glucose (5:1) and L-proline:malic acid (1:1) were the most effective, with TPC values of 19.32 ± 0.08 mg GAE/g OP and 21.64 ± 0.05 mg GAE/g OP, respectively[23].

In the present work, the extract ExtNADES3 exhibited the highest TPC (16.78 ± 0.06 mg GAE/g dry OP), followed by ExtNADES2 (15.12 ± 0.85 mg GAE/g dry OP), while ExtNADES1 showed a significantly lower TPC (12.60 ± 0.55 mg GAE/g dry OP). These findings are in line with previous studies, such as those by Ozturk et al. (2018) and Gómez-Urios et al. (2022), highlighting the potential of NADES as green solvents for efficient bioactive compound extraction.

It is also crucial to optimize extraction conditions, such as solvent composition, temperature, solid-to-liquid ratio, and extraction time. This optimization ensures higher yields of bioactive compounds and maximizes antioxidant activity. Previous studies have already highlighted the importance of these factors in improving the efficiency of phenolic extraction using NADES. Ozturk and colleagues [19] identified the optimal extraction conditions for choline chloride:ethylene glycol (1:4) as 10 wt.% water, a temperature of 60°C, a S/L of 1:10, and an extraction time of 100 minutes. Similarly, in the present study, optimal conditions were established for two NADES. For NADES1, the best extraction performance was observed at 50 °C, with ratio of 1:10 or 1:20 and an extraction time of 180 minutes. For NADES2, optimal conditions were 50 °C, a ratio of 1:20, and an extraction time of 180 minutes.

To assess the potential applicability of the developed NADES-based extracts in topical formulations, it is essential to evaluate their biological activity. In this work, L929 cells were selected to evaluate the cytotoxic and antioxidant effects of the extracts, given their relevance in testing compounds intended for topical application and systemic use. The L929 cell line is widely used as a standard model in cytotoxicity and biocompatibility studies due to its high sensitivity to toxic agents [24].

Both studied NADES-based extracts (ExtNADES1 and ExtNADES2) showed ability to inhibit ROS induced by H_2O_2, with inhibition levels comparable to 10 mg/mL ascorbic acid, a well-known antioxidant. Also, in cell viability assay post ROS treatment, extracts showed better cell viability than the positive control, suggesting that ExtNADES1 and ExtNADES2 not only provide effective ROS inhibition but also demonstrate lower cytotoxicity under these conditions.

This indicates that both NADES-based extracts possess substantial antioxidant activity, likely contributing to their potential protective effects.

To further demonstrate the superiority of the extracts, future studies could include long-term antioxidant stability tests, anti-inflammatory and antimicrobial activity evaluations and skin permeation assays. These additional studies would not only reinforce the biological relevance of the extracts but also expand their potential applications.

5. Conclusion

This study demonstrated the potential of natural deep eutectic systems as efficient and sustainable alternatives to conventional solvents for the extraction of phenolic compounds from

orange peel, an abundant agrifood waste in Portugal. Among the extracts tested, ExtNADES3 exhibited the highest TPC, while ExtNADES1 and ExtNADES2 showed superior antioxidant activity compared to the conventional extract. The optimization of extraction parameters, including temperature, extraction time, and solid-to-liquid ratio, proved to be essential for maximizing extraction efficiency.

In vitro assays, using L-929 cells, further supported the biocompatibility and antioxidant potential of the NADES-based extracts. Both ExtNADES1 and ExtNADES2 not only effectively inhibited ROS induced by hydrogen peroxide, but also maintained cell viability following ROS inhibition, in contrast to the positive control.

These findings support the potential application of NADES-based extracts in topical formulations or other health-related products, contributing to the valorization of citrus waste and the development of greener extraction technologies.

6. References

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