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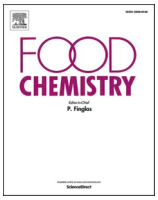

Comparison of green solvents for the revalorization of orange by-products: Carotenoid extraction and *in vitro* antioxidant activity

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ABSTRACT

Orange peels contain a considerable number of bioactive compounds such as carotenoids, that can be used as ingredients in high-value products. The aim of this study was to compare orange peel extracts obtained with different green solvents (vegetable oils, fatty acids, and deep eutectic solvents (DES)). In addition, the chemical characterization of a new hydrophobic DES formed by octanoic acid and L-proline (C8:Pro) was performed. The extracts were compared in terms of carotenoid extraction, antioxidant activity by three methods, color, and environmental impact. The results confirmed that the mixture of C8:Pro is a DES and showed the highest carotenoid extraction (46.01 μg/g) compared to hexane (39.28 μg/g). The antioxidant activity was also the highest in C8:Pro (2438.8 μM TE/mL). Finally, two assessment models were used to evaluate the greenness and sustainability of the proposed extractions. These results demonstrated the potential use of orange peels in the circular economy and industry.

1. Introduction

Worldwide, 70 million oranges are produced annually, of which the peel takes up between 40 and 50 % of the entire weight (Viñas-Ospino et al., 2023a). Orange peel contains a high level of bioactive compounds and pigments such as carotenoids (Tahir et al., 2023; Murador et al., 2019). In addition, the food industry has focused on natural additives, including natural colorants and bioactive compounds (Nabi et al., 2023). Color is the most appealing aspect of food because it increases consumer acceptance of the food. Natural colors from vegetables are increasingly being used due to their potential health benefits and customer concerns about the toxicity of synthetic colors. Carotenoids are particularly significant due to their nutritional value, in addition to their color-related properties, they have a high nutritional value for food products (Luzardo-Ocampo et al., 2021). However, the use of natural pigments in foods is limited due to their low bioavailability, stability issues, and mild health effects (Luzardo-Ocampo et al., 2021).

The preservation of carotenoid bioactivity during the extraction process is a crucial factor for the industry to consider (Saini & Keum, 2018). One alternative for carotenoid extraction is the use of green solvents. These solvents have been shown to preserve the structural integrity and bioactivity of carotenoids due to their mild extraction conditions (Stupar et al., 2021; Viñas-Ospino et al., 2023a; Viñas-Ospino et al., 2023b). Green solvents are considered alternatives to organic solvents. Various green solvents, including supercritical fluids, vegetable oils, ionic liquids, deep eutectic solvents (DES), and terpenoids, are utilized for carotenoid extraction (Boukroufa et al., 2015; Goula et al., 2017). Hydrophobic DES, composed of fatty acids or terpenoids, have the ability to dissolve natural products with limited water solubility and protect them during storage and exposure to high temperatures (Popović et al., 2022). They have demonstrated a great capacity to stabilize bio compounds, which opens up intriguing possibilities for the development of new functional foods (Morgana et al., 2022). Also, vegetable oils offer improved solubility of lipophilic substances such as carotenoids and no significant loss or degradation of carotenoids occurs during the extraction process (Portillo-López et al., 2021). Several vegetable oils, including sunflower, peanut, gingelly, mustard, sesame, palm, soybean, coconut, flaxseed, corn, canola, olive, grape, and rice bran oils have

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been tested as substitute solvents for the recovery of carotenoids (astaxanthin, lycopene, and β-carotene) from natural sources (Baria et al., 2019; Chutia & Mahanta, 2021; Han et al., 2022).

Accordingly, the present study aimed to compare the ability of different green solvents (DES, vegetable oils, and fatty acids) to extract carotenoids and antioxidant activity from orange peels, in line with the principles of Green Chemistry and the Sustainable Development Goals (Goal 11; Sustainable cities and communities, and goal 12; Responsible consumption and production). Additionally, a new mixture made of octanoic acid and L-proline was introduced. Finally, the assessment of the environmental impact, cost of the extraction process and practicability for its use on an industrial scale was evaluated. The present study is novel because there has not been a reported comparison between different green solvents and analyzed in terms of extraction efficiency, environmental impact, and price. The results obtained in this study show an enormous potential for future applications as a functional ingredient or food additive. This is due to the following factors:

- The solvents used are made of non-volatile components. As a result, they do not need to be separated from the samples and can be added directly to bioactive formulations. This approach avoids the challenges and energy requirements associated with recovering the carotenoids using an antisolvent.
- The solvents used in this study can be utilized to create formulations that may be applied in the food industry as carriers of natural carotenoids. These solvents are made of edible and Generally Recognized As Safe components, that are already Approved by the Food and Drug Administration and the European Commission as food colorants/dyes and are currently used as food additives or incorporated in food products.
- Finally, terpenoids and fatty acids, which are oily components, can improve the stability and the bioavailability of natural carotenoids in humans. Therefore, they could serve as stabilizing agents for natural carotenoids obtained from orange peels.

2. Materials and methods

2.1. Chemicals and reagents

Menthol (purity > 99 %), lauric acid (purity > 98), octanoic acid (purity > 99 %), camphor (purity > 99 %) and eucalyptol (purity > 99 %) were purchased from Sigma (St. Louis, MO, USA). L-proline was purchased from Guinama (Valencia, Spain). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DDPH), 2,2′-azino-bis (3-ethylbenzothiazolin-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich (Missouri, USA). Hexane, methanol, ethanol, and acetonitrile were purchased from J.T. Baker Chemical Co. (Deventer, The Netherlands). The pomace olive oil (OO) (Jaén, Spain) and refined sunflower oil (SO) (Córdoba, Spain) were purchased from a local supermarket in Valencia, Spain.

2.2. Raw samples

The orange samples (*Citrus sinensis*) were obtained from a local agricultural cooperative (Carlet, Spain). Oranges at the stage of commercial maturity were randomly collected between November and March (2022–2023) and immediately transferred to the laboratory for analysis. The oranges were washed with distilled water and then, the peels, were processed in a grinder and stored at − 20 °C in tightly closed packages until further use.

2.3. DES preparation

The DES were obtained using the approach described by Dai et al. (2015), with minor modifications. Each component was added in specific molar ratios (Table 1) and agitated in a water bath at 60–80 °C until a translucent liquid was formed.

Table 1
Used solvents, molar ratios, and price.

Solvent	Abbreviation	Molar ratio	Price (€/kg)*
Hexane		–	38.7*
Octanoic acid: Proline	C8: Pro	4:1	62.3*
Lauric acid: Octanoic acid	C12:C8	1:3	58.0*
Octanoic acid	C8	–	59.8*
DL-Menthol: Camphor	Me: Cam	1:1	140.0*
DL-Menthol: Eucalyptol	Me: Eu	1:1	184.0*
Sunflower oil	SO	–	3.5**
Olive oil	OO	–	4.9**

Prices were estimated according to the website of *Merk (Germany) and **local supermarket.

2.4. Extraction procedure

In brief, 1 g of fresh orange peels was mixed with 20 mL of solvent (DES, vegetable oil, fatty acids, or hexane). The extractions were conducted in triplicate by Ultrasound Assisted Extraction (UAE) for 20 min by an ultrasonic processor Q500 (Qsonica, USA) with ultrasound intensity of 60 % (120 W) at 45 °C. These conditions were chosen based on our previous article (Viñas-Ospino et al., 2023a). The resulting mixture was filtered and stored in amber vials at 4 °C until analysis.

2.5. Extracts characterization

2.5.1. Total carotenoids

The total carotenoid (TC) content of orange peels was quantified at 450 nm using a Genesys 10S UV–Vis spectrophotometer (Thermo Fisher Scientific, USA). The correspondent solvent was used as a blank in each extract. The TC was calculated using the Beer-Lambert law and hexane absorption extinction coefficient (2560 L mol^{-1} cm^{-1}). The total carotenoid content of orange peels was reported as μg β-carotene/g of extract.

2.5.2. Total antioxidant capacity (DPPH)

The assay was conducted according to the method of Brand-Williams et al. (1995). Briefly, 1.45 mL of DPPH was combined with 50 μL of an appropriate sample dilution and incubated at room temperature for 30 min. The absorbance at 515 nm was measured using a Lambda 365 spectrophotometer (PerkinElmer ® Massachusetts, USA). A Trolox standard was made in the range of 0–500 μM to create the calibration curve, and the results were reported as μM TE (Trolox Equivalent)/mL of sample.

2.5.3. Trolox equivalent antioxidant capacity (TEAC)

To measure TEAC, the method of Re et al. (1999) was used. First, the ABTS radical (ABTS•+) (7 mM) was produced in 440 μL of potassium persulfate (140 mM) which was held in the dark at room temperature for 12–16 h. The solution was diluted with ethanol until an absorbance of 0.70 ± 0.02 was obtained at 734 nm. Then, 2 mL of the generated ABTS•+ was mixed with 100 μL of sample and incubated at 30 °C for 3 min before measuring it. The calibration curve utilized a Trolox standard within the range of 0–250 μM. The results were reported as μM TE/mL of sample.

2.5.4. Ferric Reducing-Antioxidant power (FRAP)

The FRAP test was performed with some modifications to the procedure outlined by Gardeli et al. (2008). The FRAP reagent solution was prepared daily by combining the TPTZ radical solution (10 mM in solution with 40 mM of HCl), ferric chloride hexahydrate solution (20 mM), and acetate buffer (300 mM, pH 3.6) were combined in a 1:10 (v/v) ratio. Next, 900 μL of FRAP solution, 90 μL of distilled water, and 30 μL of adequately diluted sample were incubated in amber glass tubes at

37 °C. The calibration curve for the FRAP standard was performed under identical conditions as the samples, with concentrations ranging from 0 to 250 μM. The results were reported as μM TE/mL of sample.

2.5.5. Color determination

Color measurements were conducted using a Hunter Labscan II spectrophotometric colorimeter (Hunter Associates Laboratories Inc., Reston, VA., USA) following the guidelines of the *Commission Internationale de d'Eclairage* (CIE 2004) and utilizing the CIELAB color system (L*, a*, b*). The CIELAB color space is represented by three scalar parameters or Cartesian coordinates: L* (lightness), which ranges from 0 (absolute darkness) to 100 (absolute white); a* (redness), b* (yellowness-blueness). The analysis also considered the color difference (ΔE) compared to the standard (hexane extract).

2.6. Octanoic acid: L-proline characterization

As one of the objectives of this study was to introduce octanoic acid: proline (4:1) as a novel solvent, the mixture was characterized to demonstrate the presence of interactions between its two components and to confirm the formation of a DES.

2.6.1. Nuclear magnetic resonance (NMR) measurements

The DES (300 μL) and pure compounds (10 mg) were dissolved in 200 μL and 500 μL, respectively of $CDCl_3$ (99,80 % D, Eurisotop). The ^1H NMR and ^1H–^1H NOESY spectra were acquired on a Bruker Advance 400 at 400 MHz using a 5 mm NMR tube. The signals of the DES and individual compounds signals were assigned using MestReNova 11.0 software (Mestrelab Research, Spain). Chemical shifts (δ) are reported in ppm.

2.6.2. Polarized optical microscopy (POM)

A drop of DES and the physical mixture was placed in a microscope glass slide at room temperature and observed by a transmission mode of an Olympus BX-51 polarized optical microscope (coupled to an Olympus KL2500 LCD (Tokyo, Japan) cold light source. The images were obtained with a digital camera (Olympus DP73, Tokyo, Japan) connected to the microscope, and then the Olympus Stream Basic 1.9 software (Olympus, Tokyo, Japan) was used to treat the images.

2.6.3. Fourier transform infrared spectroscopy (FTIR) analysis

FTIR spectroscopy of the DES and their pure constituents was conducted using a Spectrum Two spectrometer (Perkin Elmer S.L., Madrid, Spain) to obtain the infrared spectra. The samples were scanned from 4000 to 450 cm^{-1} at a resolution of 16 cm^{-1}. All measurements were performed at room temperature.

2.7. Green metrics

The environmental impact was assessed using the EcoScale proposed by Van Aken et al. (2006). Based on multiple criteria and the calculated Penalty Points (PP) values, this approach considers the PP to be subtracted from a 100 % ecologically safe process. The EcoScale considers factors including the usage of hazardous solvents, energy consumption, yield, and financial considerations. The different solvents used in this study were compared for the carotenoid extraction process. The scores used to rate the outcomes were as follows: excellent (>75), satisfactory (>50), and inadequate (<50). Then, the Blue Applicability Grade Index (BAGI) proposed by Manousi et al. (2023) was used to evaluate the practicality of the total carotenoid determination using green solvents. This metric tool assigns a value between 25 and 100 (the higher the number, the more useful the approach).

2.8. Statistical analysis

The experiments were performed in triplicate and the results are presented as means and standard deviation. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, California, USA). Mean differences were compared using analysis of variance (ANOVA) with a significance level of 0.05. Additionally, Tukey's test ($p < 0.05$) was used to analyze significant differences in factors with more than two levels. The intercorrelations among the studied parameters were analyzed using of Pearson correlation and *p*-value.

3. Results and discussion

3.1. Solvents selection

For the present study different green solvents were used, including DES, vegetable oils and fatty acids. It is worth noting that DES are the only solvents that require prior preparation with specific molar ratios and conditions. Table 1 presents the solvents utilized, their abbreviations, molar ratios, and prices. The DES; Me:Eu, Me:Cam and C12:C8 have been previously reported and characterized as DES (Morgana et al., 2022; Rodrigues et al., 2020; Sportiello et al., 2023; Viñas-Ospino et al., 2023a; Viñas-Ospino et al., 2023b). These DES were selected based on the previous article (Viñas-Ospino et al., 2023a) and due to their promising carotenoid extraction efficiency. However, it should be noted that C8:Pro is a novel mixture that has not been previously reported as a DES. One of the aims of this study was to characterize this mixture and introduce it as a new solvent for carotenoid extraction. Additionally, the extraction using only octanoic acid was performed to compare the extraction efficiency when it is mixed with L-Proline. For vegetable oils, olive oil and sunflower oil were chosen as they have been reported as good alternatives for carotenoid extraction in other matrices and their relatively low price (Ordóñez-Santos et al., 2015; Chutia & Mahanta, 2021).

3.2. Carotenoid extraction efficiency

The extraction conditions were chosen based on our previous article (Viñas-Ospino et al., 2023a). Larger concentration differences between phases can be produced with a higher solvent-to-solid ratio, which can improve the penetration of carotenoids into the medium and increase mass transfer. In addition, longer extraction times and high temperatures can increase oxidative degradation and isomerization of carotenoids (Ordóñez-Santos et al., 2015). Therefore, the extraction temperature used in this study was kept below 45 °C and the extraction time was 20 min. Carotenoids are commonly extracted using organic solvents because of their hydrophobic nature (Saini & Keum, 2018; Murador et al., 2019). In the present study hexane was used as control and the data depicted in Fig. 1 shows that hexane yielded 39.28 ± 2.05 μg/g of TC, which is similar to the value reported by Murador et al. (2021), who reported 53.73 ± 5.20 μg/g from orange peel extracts. The amount of carotenoids present depends on several factors including extraction conditions, like orange species, harvesting season and site, as well as storage circumstances (Anticona et al., 2021).

Terpene-based and fatty acid-based DES, vegetable oils and pure fatty acids were examined as potential substitutes for conventional organic solvents in the extraction of carotenoids from orange peels. The results of the TC content using UAE and different solvents are displayed in Fig. 1. The yields varied depending on the type of solvent ranging from 46.01 μg/g (C8:Pro) to 19.89 μg/g (Sunflower Oil). It can be observed that DES efficiently extracts more carotenoids than vegetable oils and pure octanoic acid. This effect can be attributed to the fact that viscosity of hydrophobic DES is lower than vegetables oils, thereby promoting the diffusion of the target compounds (Portillo-López et al., 2021). OO showed a higher carotenoid extraction than SO as previously reported by Chutia & Mahanta (2021). This author concluded that olive oil as a solvent was found the best solvent for the extraction of carotenoids from passion fruit. Which could be explained due to the higher amount of short chain fatty acids in OO compared to SO and this helps

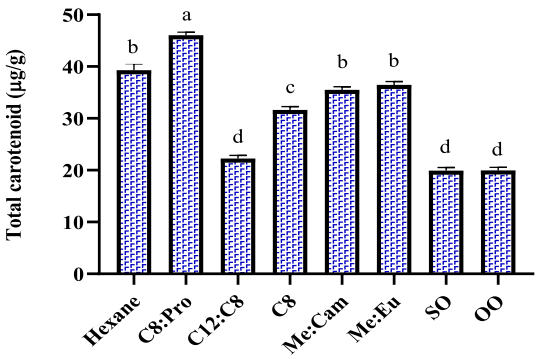


Fig. 1. Total carotenoid content (µg/mL) in orange peel extracts with different solvents. Carotenoid content was expressed as the means (n = 3) ± SD. The statistical analysis reveals significant differences ($p < 0.05$) between values denoted by different lower-case letters (a-d) are significantly different as determined by Tukey ' s honestly significance difference (HSD). C8: Octanoic acid, Pro: Proline, C12: Lauric acid, Me: menthol, Cam: Camphor, Eu: Eucalyptol, SO: Sunflower oil, OO: Olive oil, (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

enhance the extraction of carotenoids (Chutia & Mahanta, 2021). The C8:Pro (4:1) mixture was the most efficient solvent for extracting carotenoids, yielding more than hexane extraction. Pure octanoic acid (31.61 ± 1.2 µg/g) was also used for the extraction. When octanoic acid was mixed with L-Proline the yield significantly increased (46.01 ± 1.4 µg/g), suggesting a potential interaction between these compounds.

The new mixture reported in the present study formed by octanoic acid and L-proline demonstrated a promising extraction yield. The extraction efficiency of the target molecules is strongly influenced by the physicochemical properties of the solvents (Stupar et al., 2021). When L-Proline is mixed with octanoic acid an equilibrium between the hydrophobicity of octanoic acid and proline is achieved. As reported by Murador et al. (2021) orange peels contain polar carotenoids such as β-cryptoxanthin, lutein and zeaxanthin. These carotenoids have polar ionone rings, and they have different interactions with the membrane lipids than β-carotene (Stupar et al., 2021). As a result, xanthophylls may interact more effectively with carboxyl groups from fatty acids-based eutectics, better than menthol-based eutectics.

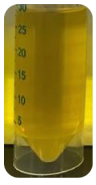
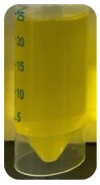
3.3. Total antioxidant capacity and color determination

The evaluation of the antioxidant capacity in the extracts obtained by different solvents was conducted considering the potential of carotenoids as antioxidants. A proper selection of methods is necessary to determine the total antioxidant capacity based on the reaction properties of the compounds. There is no single accurate method to assess antioxidant activity that reflects the impact of all antioxidants present in a complex combination of bioactive molecules due to the various mechanisms of action and the nature of antioxidant and oxidizing compounds (Gómez-Urios et al., 2023). Three antioxidant methods, namely DPPH, ABTS and FRAP were employed in this study and the results are presented in Table 2. The C8:Pro mixture exhibited the highest antioxidant capacity across all three methods. Furthermore, the values obtained through the DPPH method were the highest. This could be attributed to the fact that the DPPH radical is only soluble in organic solvents, thereby measuring the antioxidant capacity of more nonpolar components such as carotenoids.

In all three antioxidant capacity methods, DES extracts exhibited greater antioxidant activity. Previous studies have reported that DES has intrinsic antioxidants and this increases the final antioxidant activity in the extract (Cvjetko Bubalo et al., 2015; Mitar et al., 2019; Panić et al., 2021). However, the choice of DES type and molar ratios can affect the extraction yields, resulting in varying antioxidant capacities (Fuad et al., 2021). Additionally, the OO extract exhibited high antioxidant activity using ABTS and FRAP methods. This can be attributed to the inherent antioxidant activity of OO (Civan & Kumcuoglu, 2019). The observed variation in results between the methods can be attributed to their distinct mechanisms of action. The DPPH radical could be decreased by reactions with antioxidant compositions that can donate hydrogen, producing the non-radical form. Conversely, The TEAC assay utilizes ABTS•+ to determine hydrophilic and lipophilic antioxidant compounds in samples after a reaction time of approximately 30 min (Anticona et al., 2021). The variation in antioxidant activity may have been also influenced by other lipophilic phenolic compounds and natural antioxidants, such as tocopherols (Baria et al., 2019).

Regarding the color characteristics that influence consumer acceptance (Benvenutti et al., 2022), the CIELAB scalar coordinates (L*,a*,b*) were measured and the results are presented in Table 2. Significant differences ($p < 0.05$) were found in the color parameters between the extracts obtained with different solvents and the hexane extract. Table 2 also shows the visual appearance of the obtained extracts. The colors of the extracts containing carotenoids exhibited light orange and luminous

Table 2
Color characterization, visual appearance, and antioxidant activity in orange peel extracts with different solvents.

	Hexane	C8:Pro	C12:C8	C8	Me:Cam	Me:Eu	SO	OO
								
Color								
L*	73.4 ± 1.3^{c}	71.6 ± 1.2^{d}	76.7 ± 0.5^{a}	74.3 ± 0.6^{b}	76.0 ± 0.5^{a}	76.2 ± 1.2^{a}	76.8 ± 1.1^{a}	70.7 ± 1.0^{d}
a*	0.98 ± 0.0^{a}	0.92 ± 0.2^{a}	−9.26 ± 0.4^{d}	−8.10 ± 0.4^{c}	−8.38 ± 0.4^{c}	−9.22 ± 1.1^{d}	−8.02 ± 0.5^{c}	−6.53 ± 0.5^{b}
b*	95.2 ± 2.1^{a}	76.2 ± 2.0^{b}	48.4 ± 1.6^{e}	60.9 ± 1.6^{d}	66.4 ± 1.9^{c}	67.9 ± 2.0^{c}	46.5 ± 1.2^{e}	68.1 ± 2.3^{c}
ΔE	0	10.5 ± 0.3^{e}	26.9 ± 0.4^{a}	21.3 ± 1.6^{b}	17.9 ± 2.3^{d}	17.42 ± 0.1^{d}	27.2 ± 0.3^{a}	18.8 ± 0.7^{c}
DPPH (µM TE/mL)	944.3 ± 13.1 ^{g}	2438.8 ± 21.2^{a}	1322.4 ± 14.7^{e}	1340.4 ± 18.3^{e}	1934.6 ± 15.3^{b}	1664.5 ± 19.0^{d}	1250.4 ± 14.5^{f}	1790.6 ± 17.7^{c}
ABTS (µM TE/mL)	355.1 ± 4.5 ^{g}	1057.3 ± 18.3^{a}	436.1 ± 8.4^{f}	463.1 ± 6.5^{d}	445.1 ± 7.7^{e}	508.2 ± 12.2^{c}	400.1 ± 3.7^{e}	661.2 ± 4.8^{b}
FRAP (µM TE/mL)	854.2 ± 8.5^{d}	1456.4 ± 12.2^{a}	612.4 ± 6.2 ^{g}	743.41 ± 2.1^{f}	1124.6 ± 10.1^{b}	965.7 ± 2.3^{c}	840.3 ± 10.9^{e}	990.6 ± 11.4^{b}

L*,lightness, a*, associated with changes in redness, and b*, associated with changes in yellowness blueness. C8: Octanoic acid, Pro: Proline, C12: Lauric acid, Me: menthol, Cam: Camphor, Eu: Eucalyptol, SO: Sunflower oil, OO: Olive oil, DPPH: 2,2-Difenil-1-Picrilhidrazilo, ABTS: (2,2′–azino–bis–(3–ethylbenzothiazoline–6–sulphonic acid), FRAP: ferric reducing antioxidant power. The results are expressed as the means ± SD (n = 3). Presented values followed by different lower-case letters (a-g) are significantly different ($p < 0.05$) as measured by Tukey's test.

yellow. Overall, the samples had light colors with a lightness (L*) ranging from 70.7 to 76.8. Additionally, it was observed that nearly all the samples were located in the second quadrant, which corresponds to negative values of a*, while the values of b* were positive. The most positive a* values were associated with red and dark color, which corresponded to hexane and C8:Pro extract. The positive values of b* indicate a yellowing of the extracts, which is related to the carotenoid content (Baria et al., 2019; Ramos-Escudero et al., 2019). The visual appearance of C8:Pro and hexane extract differs from the other extracts, which is also evident in the ΔE results. The color difference indicates a significant difference (>3) between all the extracts compared to the standard (hexane extract). The highest ΔE was observed in C12:C8 and sunflower oil ($p < 0.05$), while the lowest color difference was observed in C8:Pro extract. This difference may be attributed to the carotenoid content, which is the highest in the C8:Pro extract, as previously mentioned.

3.4. Correlation between the studied variables

The Pearson correlation coefficient demonstrated the correlation between carotenoid content, antioxidant activities (measured by DPPH, ABTS and FRAP assays), and color coordinates (L*, a*, b*, ΔE) as shown in Fig. 2. The results showed a statistically significant correlation ($p < 0.05$) between carotenoid content and FRAP (r = 0.640); as well as color coordinates.

a* (r = 0.638) and b* (r = 0.710). However, a low correlation was observed between carotenoid content, DPPH (r = 0.408) and ABTS (r = 0.432). The discrepancies in antioxidant activities against different free radicals can be attributed to the diverse functional groups and structures of the compounds. These results may be linked to the fact that carotenoids are not the only bioactive components of orange peel extracts that contributed to the total antioxidant activity and that multiple methodologies might be employed to define antioxidant activity, thus, bioactive components and antioxidant activity did not always display a strong association (Civan & Kumcuoglu, 2019). The antioxidant activity of the orange peel extracts is an intricate process that heavily depends on the extraction technique.

3.5. C8:Pro characterization

The mixture of octanoic acid and L-proline yielded highly promising results in terms of its ability to extract carotenoids and antioxidant capacity. This new mixture combines two ingredients found in food products that may have an affinity for carotenoid extraction. L-proline, a non-polar amine, has hydrophobic properties and therefore a greater affinity to extract hydrophobic compounds. Studies have shown that L-proline can improve carotenoid extraction efficiency when combined with other solvents or techniques. For instance, it has been used as an additive or co-solvent in extraction methods to enhance the solubility and stability of carotenoids. L-proline acts as a complexing agent, facilitating the release of carotenoids from the cellular matrix and increasing their extraction yield (Gómez-Urios et al., 2022; Karadendrou et al., 2022). Additionally, L-proline has antioxidant properties that can help protect carotenoids from degradation during extraction procedures, thereby preserving their bioactivity. Its natural abundance in various plant sources makes it an appealing choice for sustainable extraction procedures (Zhuang et al., 2017). In contrast to previously reported hydrophobic DES, menthol-based DES may exhibit some organoleptic characteristics such as flavor or aroma, which could limit their applicability in specific products (Rodrigues et al., 2020; Pitacco et al., 2022) and these DES are relatively expensive. Therefore, the aim of this study is to propose an alternative green solvent that can be used in a wide range of food products to enhance their bioactive and technological properties. The selection of the molar ratio is a crucial aspect in formulating a DES. For this reason, the C8:Pro mixture was also prepared in different ratios, namely 2:1 and 3:1. However, the solvent was solid at room temperature in these ratios, rendering them unsuitable for the extraction of bioactive compounds. The subsequent section focuses on the characterization of the C8:Pro (4:1) mixture to investigate the presence of molecular interactions and the formation of a new DES.

Several NMR experiments were performed, to determine whether the 4:1 M ratio proportion of octanoic acid and proline produced a DES. Fig. 3 shows the NMR results, which included proton NMRs for each individual DES component, (C8 and Pro) and for the DES and physical mixture (mixture of octanoic acid and proline without forming a DES

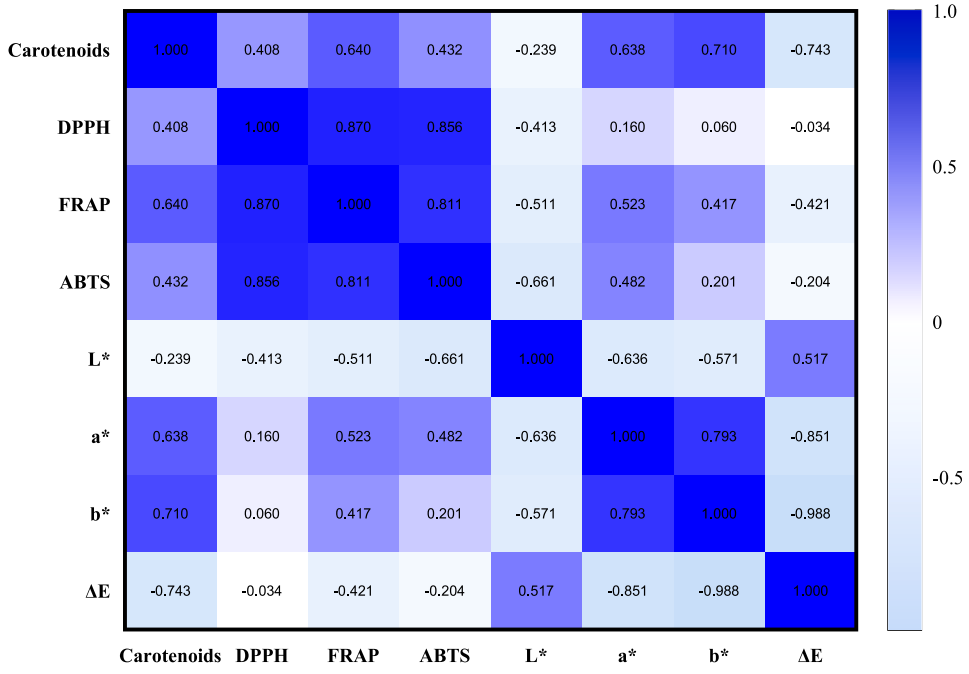


Fig. 2. Heat map of the intercorrelation between the studied parameter using the Pearson correlation coefficient (r) of the extracts obtained by different solvents. L*, lightness, a*, associated with changes in redness, and b*, associated with changes in yellowness to blueness, DPPH: 2,2-Difenil-1-Picrilhidrazilo, ABTS: (2,2′–azino–bis–(3–ethylbenzothiazoline–6–sulphonic acid), FRAP: ferric reducing antioxidant power.

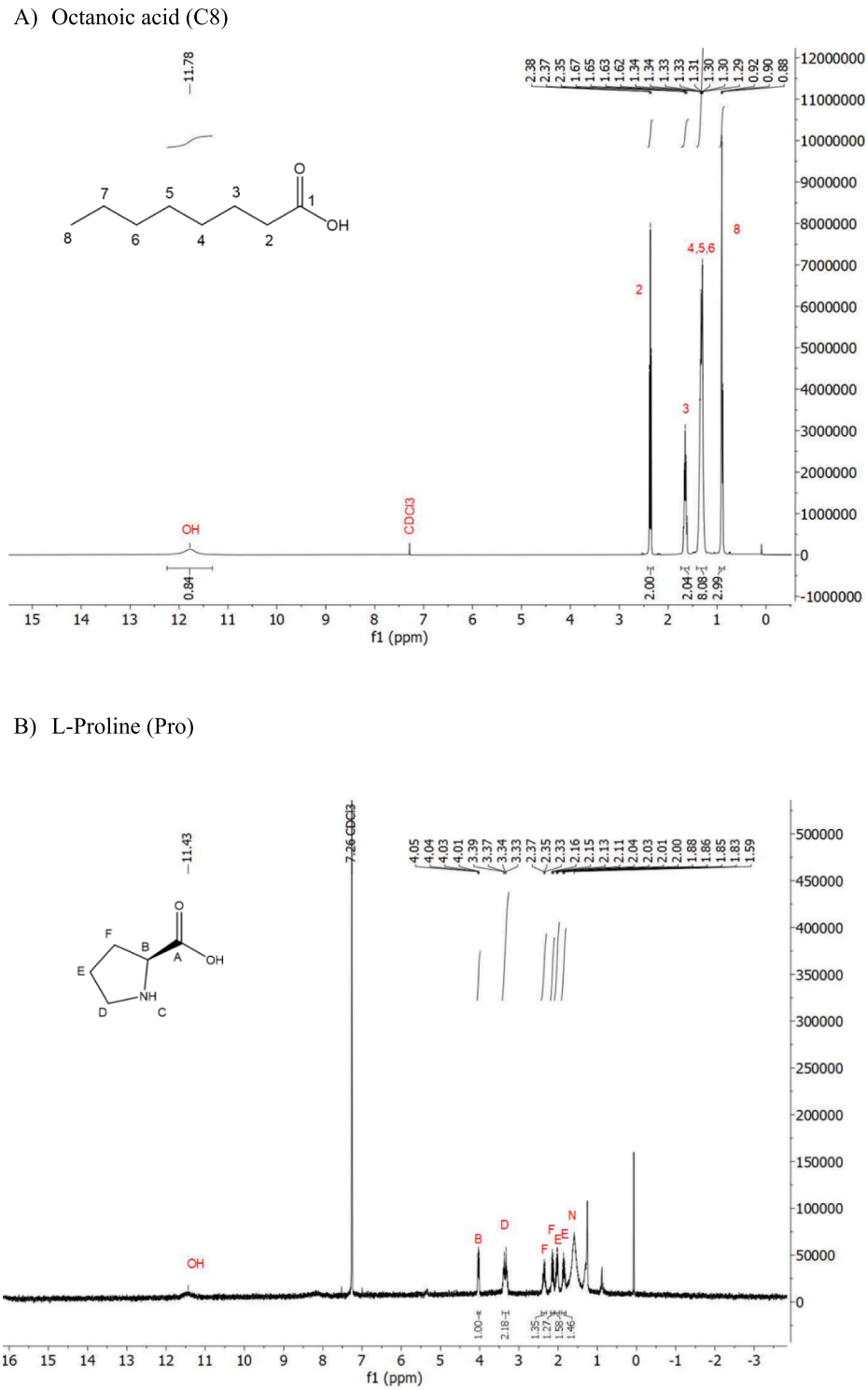
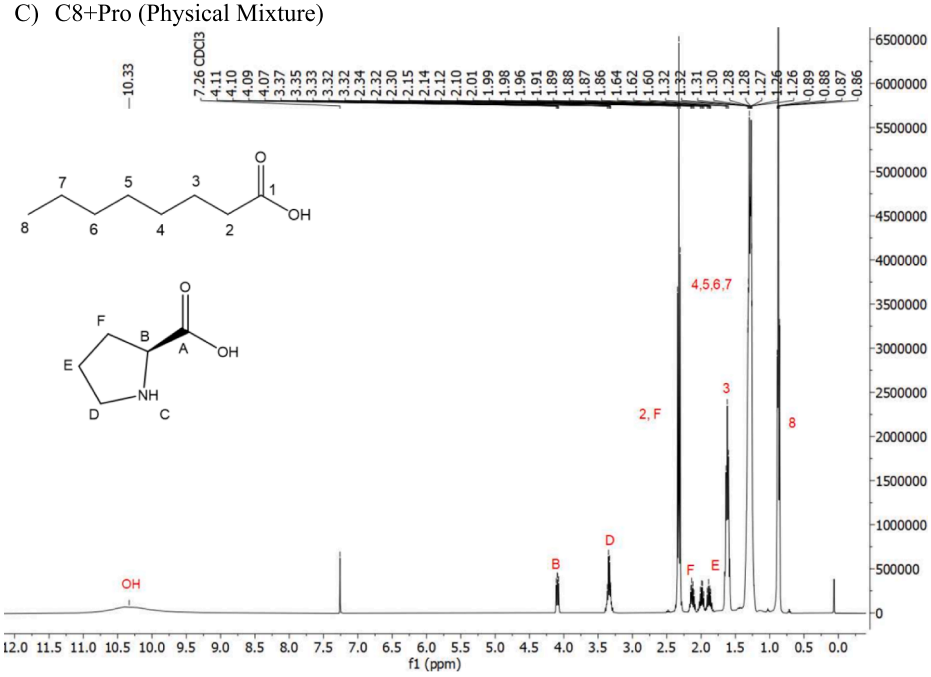


Fig. 3. ^{1}H NMR spectra and signal assignment of A) Octanoic acid, B) L-proline, C) C8:Pro) physical mixture, D) C8:Pro DES. C8: Octanoic acid, Pro: Proline, DES: Deep eutectic solvent.

through heating and stirring). Using the individual ^{1}H proton NMR of C8 and Pro, all signals were identified in both physical mixture and DES ^{1}H spectra. Significant changes were observed by comparing the chemical shifts in the physical mixture and DES spectra. While in the physical mixture the OH group from both C8 and Pro appeared at δ10.36 ppm, they appeared separately at δ 11.83 and δ 9.35 ppm, respectively, in the DES ^{1}H spectrum. All the signals from C8 in the physical mixture matched those present in the individual spectrum. However, in the DES sample these signals shifted to lower δ values indicating an intermolecular interaction. Additionally, the NH signal from Pro (δ 1.62 ppm) is not visible on the C8 + Pro (physical mixture) spectrum due to overlap with the signal from C8. However, due to the shifting on the C8 signals it is possible to observe that there is no NH signal at ~ δ 1.62 ppm thus supporting the theory of DES formation. Furthermore, 2D experiments, specifically NOESY, was conducted on the DES sample to identify interactions between C8 and Pro and the corresponding results are

C) C8+Pro (Physical Mixture)



D) C8:Pro (DES)

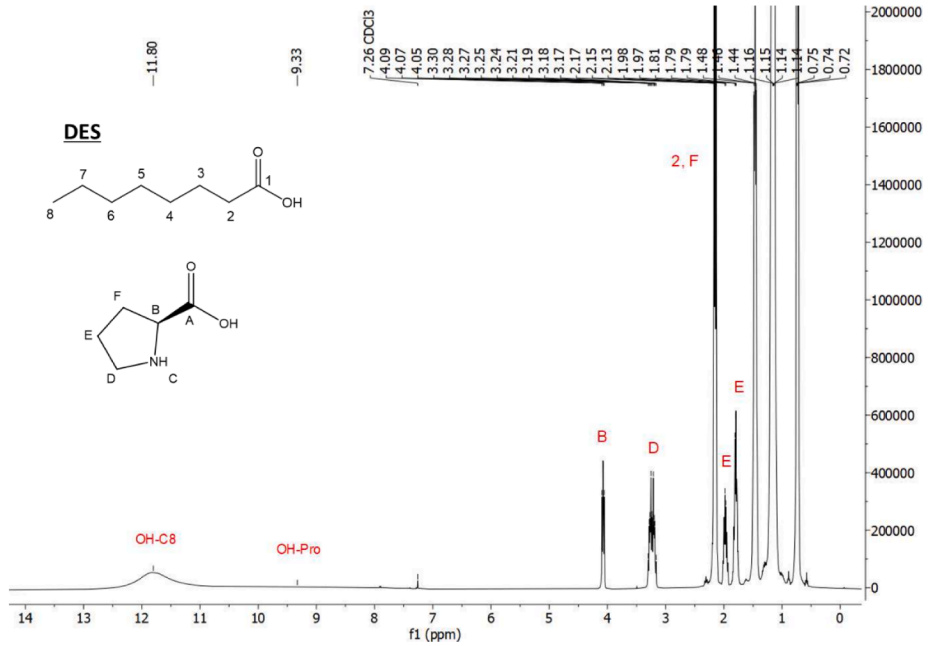


Fig. 3. (*continued*).

displayed in Fig. 4a. It was not possible to directly observe interactions between the two molecules due to signal overlap.

Then, polarized optical microscopy (POM) was also used to confirm the formation of the DES. The absence of crystals or powder in the images presented in Fig. 4b (1 and 2) indicates a homogeneous mixture, confirming that C8:Pro (4:1) is indeed a new DES. The physical mixture was also analyzed by this technique and Fig. 4b (3 and 4) revealed suspended particles of proline for comparison.

Molecular interactions have been extensively examined using FTIR spectroscopy, particularly for researching the development of hydrogen bonds between the components of DES (Huang et al., 2022). The FTIR method was employed to examine the C8:Pro system and its constituents, in Fig. 4c are shown the acquired spectra. When comparing the spectra obtained for DES with those of individual compounds, it is evident that the DES spectrum is akin to the other two spectra with slight variations. The main features of L-proline spectra comprise the characteristic peaks located at 1620 cm^{-1} and 1220 cm^{-1}. The peak at 1620 cm^{-1} corresponds to N—H bending while the one at 1220 cm^{-1}, corresponds to the C—N stretching. Different peaks are observed in the spectra of C8:Pro, which may indicate potential interactions. Further analysis confirms that the hydrogen bonds formed between the two molecules have caused the peaks to shift and broaden, supporting the

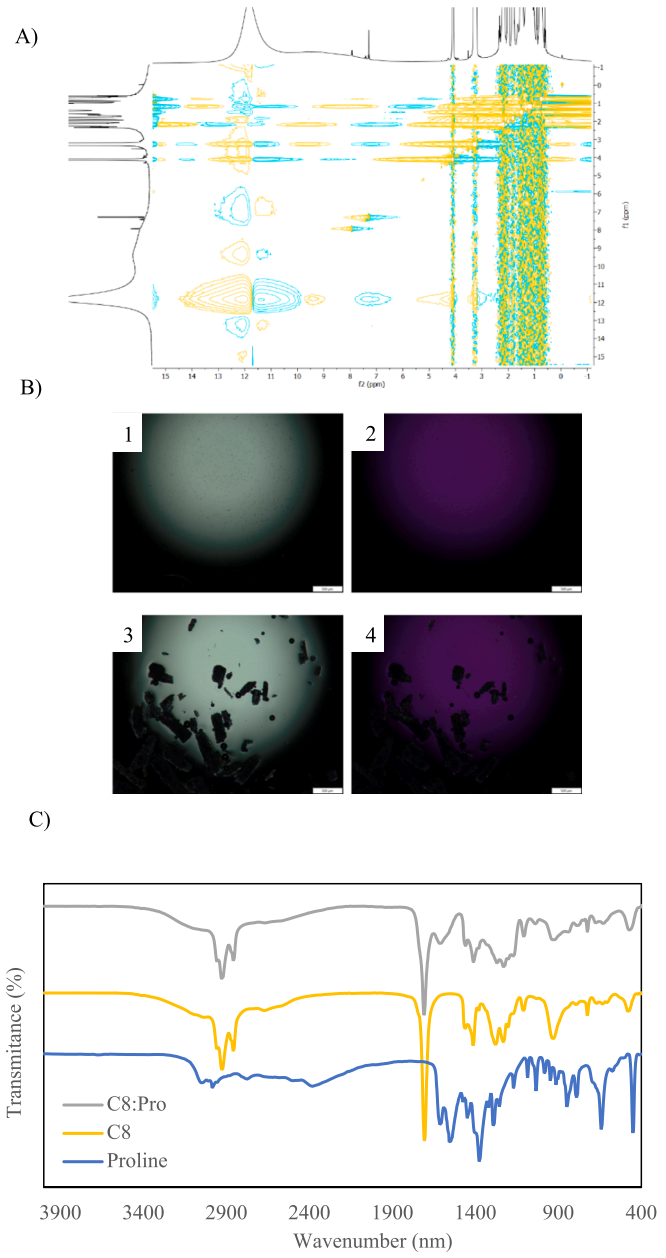


Fig. 4. ^{1}H–^{1}H-NOESY spectra and signal assignment of C8:Pro (4:1) (A), Optical microscopy of 1) DES C8:Pro (4:1), and 3) physical mixture C8 + Pro; and POM images of 2) DES C8:Pro (4:1), and 4) physical mixture C8 + Pro (B), FTIR spectra of C8:Pro and individual components (C).

idea that the components were already interconnected instead of a new complex being formed (Jurić et al., 2021). Considering all the obtained results together (NMR, NOESY, POM, and FTIR) it can propose the creation of a new deep eutectic solvent formed by octanoic acid and L-proline in a molar ratio of 4:1.

3.6. Green metrics

Green metrics were used to compare the environmental impact of the solvents used in this study and to evaluate the practicality of the method used for total carotenoid determination. Table S1 (Supplementary information) displays the results obtained through the Eco-Scale tool. The analytical Eco-Scale tool assigns penalty points (PPs) to process elements that do not conform an ideal green method. In a perfect green procedure, no waste is generated, minimal energy is used, and chemical consumption is reduced or eliminated (Chemat et al., 2019). The Eco-Scale evaluation rates the optimal green process with a score of 100. Previous studies have used this instrument and confirmed its reliability (Benvenutti et al., 2022; Viñas-Ospino, et al., 2023b). In the reported results can be observed that all the green solvents scored over 75, demonstrating exceptional reaction conditions. However, the hexane extract scored only acceptable results. The cost/efficiency parameter is crucial for industrial scale applications, and it is also taken into account in the Eco-Scale. Table 1 shows the prices of each solvent, with Me:Cam and Me:Eu being the most expensive. In contrast, vegetable oils are the most affordable option and require no prior preparation. It is noteworthy that the cost presented in Table 1 for the DES pertains to laboratory supplier, while that for the oils refers to the supermarket prices. However, the use of Me:Cam and Me:Eu resulted in an extraction efficiency for carotenoid that is almost twice as high as that of vegetable oils. The newly proposed DES, C8:Pro, yielded the most favorable outcomes for carotenoid and antioxidant content, and its price is also not significantly high. In conclusion, the results of the green metric tool provided a comprehensive and environmentally friendly profile for comparing the solvents used in this study.

Finally, the BAGI metric tool score was 72.5 and the pictogram is shown in Figure S2 (Supplementary Information). This score means that the total carotenoid determination method using the green solvents compared in this article has a good applicability potential. The BAGI metric is a tool that evaluates attributes such as the type of analysis, the number of analytes simultaneously determined, the number of samples that can be analyzed in one hour, the type of reagents and materials, among others. A value between 2.5 and 10 is then assigned to each category and an asteroid pictogram is obtained. This tool has been shown to be a good an simple tool to determine positive and negative ranges in terms of application and practicality of a method (Manousi et al., 2023).

4. Conclusion

This study is the first to compare the effects of various solvents (vegetable oils, DES, fatty acids, and hexane) for the extraction of bioactive compound from orange by-products. Furthermore, a new hydrophobic DES made of compounds present already in food products was proposed and characterized, demonstrating promising results as an extracting solvent. It is worth noting that C8:Pro extracts produced the highest yield of carotenoids and antioxidant activity. Substituting hazardous solvents with renewable options can make the extraction procedure becomes more secure, efficient, and financially feasible. As research in this field progresses, the combination of green solvents with state-of-the-art extraction techniques shows significant potential for the future of carotenoid extraction and sustainable industries as a whole. The environmental friendliness and the practicability of the process were positively evaluated by means of the Eco-Scale and the BAGI metric tools. These results provide valuable insights for the revalorization of fruit by-products. Future work should be directed towards evaluating the biological properties of the obtained extracts in cellular assays.

CRediT authorship contribution statement

Adriana Viñas-Ospino: Writing – original draft, Investigation. **Ana Rita Jesus:** Conceptualization. **Alexandre Paiva:** Supervision, Conceptualization. **Maria J Esteve:** Supervision, Funding acquisition. **Ana Frígola:** Conceptualization. **Jesús Blesa:** Writing – review & editing, Investigation. **Daniel López-Malo:** Writing – review & editing, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.138530.

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