**Optimization of the extraction techniques using Natural Hydrophobic Deep Eutectic Solvents for the recovery of biomolecules from food and food industry by-products**

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This PhD Project focused on the optimization of a green extraction technique based on Natural Hydrophobic Deep Eutectic solvents (HDESs) for the recovery of carotenoids from by-products of the vegetable food processing industry as well as an unconventional source, as algae. The implemented experimental design was structured in three steps: 1) assessment of the physicochemical properties and the extracting efficiency of several Natural HDESs for the recovery of carotenoids from different substrates; 2) selection of the best performing solvents and substrates and optimization of the extraction technique; 3) development of food and cosmetic applications of the enriched carotenoids extracts evaluating the antioxidant stability and the consumers’ acceptability of the obtained new products.

**Ottimizzazione delle tecniche di estrazione mediante Natural Hydrophobic Deep Eutectic Solvents per il recupero di biomolecole da prodotti e sottoprodotti dell’industria alimentare**

Questo progetto di Dottorato di Ricerca è stato focalizzato sull'ottimizzazione di una tecnica di estrazione verde utilizzando solventi eutettici profondi idrofobici (HDESs) per il recupero di carotenoidi da sottoprodotti vegetali dell'industria alimentare e da una fonte alimentare non convenzionale quale le alghe. Il progetto è stato strutturato in tre fasi: 1) valutazione delle proprietà chimico fisiche e dell’efficienza estrattiva di diversi HDES naturali; 2) selezione dei solventi e dei substrati più performanti e ottimizzazione della tecnica di estrazione; 3) sviluppo di applicazioni alimentari e cosmetiche degli estratti arricchiti di carotenoidi, valutandone la stabilità antiossidante e l'accettabilità da parte dei consumatori.

**Key words**: food by-products, green extraction, microalgae, natural hydrophobic deep eutectic solvents, optimization.

# Introduction

In recent years, sustainability and green engineering principles have been the ground for scientific research in many field. In the food sector, the development of sustainable and economically viable bio-based processes to obtain highly added-value compounds for functional foods and dietary supplements production is a hot topic, due to the increasing consumers’ awareness of the pivotal role played by nutrition in human health. Furthermore, also pharmaceutical and cosmetic industries have an interest in new moieties, which may be utilized in product formulation.

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| **Table 1.** *Structure of this PhD project*. |
| **Aim** | **Activities** |
| Implementation of green extraction processes for carotenoids recovery  | * Selection of suitable Natural HDESs according to the literature review;
* Selection of rich-carotenoid matrices;
* Physicochemical characterization of the selected Natural HDESs;
* Selection of the best performing solvents for each matrix.
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| Optimization of the extraction process by implementing a Box-Benkhen Design with the goal of maximizing carotenoid yield in the extracts\* | * Identification of the proper combination between HBA:HBD molar ratio, solvent to sample ratio and the optimum extraction time;
* HPLC analysis on the extracts;
* Design of a purification step.
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| Development of food and cosmetic applications for the enriched carotenoids extracts\*\* | * Production of cosmetic products and food supplements added with the obtained extracts;
* Antioxidant stability and consumer tests.
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| \* ongoing activities \*\* activities to be conducted in the last semester of this PhD project. |

Nowadays, the industrial recovery of these moieties represents a challenging step, with problems related to costs, efficiency, selectivity and environmental sustainability (Choi et al., 2019). About this latter issue, the use of Natural Deep Eutectic Solvents (NaDESs) has proved to be a potential alternative for the green extraction of natural bioactive compounds (Cvjetko Bubalo et al., 2018; Sportiello et al., 2023). NaDESs represent a subcategory of the Deep Eutectic Solvents (DESs), which are peculiar mixtures obtained by combining two or more constituents, generally solid at room temperature, with a resultant melting point depression and the transition into a liquid state. DESs can be easily prepared by mixing hydrogen bond acceptors (HBAs) and hydrogen bond donors (HBDs) in specific molar ratios (Martins et al., 2018). NaDESs are obtained when limiting the selection of the HBA and HBD to moieties derived from natural sources and, depending on the resultant polarity of the mixture, they can be hydrophilic or hydrophobic, thus able to solubilize an extensive range of molecules. Monoterpenes, carboxylic and fatty acids are the most common natural HBAs and HBDs utilized for realizing Natural Hydrophobic DESs (HDES), generally having a very low cost and negligible ecological impact and toxicity. Additionally, their high biocompatibility and their food-grade nature open the way for new direct applications of the extracts *‘as such’* in the food, cosmetic and pharmaceutical industry. Based on these assumptions, the aim of this PhD project is the optimization of a green extraction technique using Natural HDESs for the recovery of carotenoids from matrices of emerging interest, such as microalgae, and several real vegetable by-products supplied by industries located in Northeast Italy. Additionally, a further aim is the formulation of products enriched with the obtained extracts, demonstrating the potential use in industrial applications. In Table 1 are reported the activities carried out to achieve the goals of this PhD project.

1. **Materials and Methods**

# Sample and solvent preparation and physicochemical characterization

Initially, as extraction substrates were utilized by-products (peels) deriving from the industrial processing of fresh carrots, yellow and red peppers and pumpkins, and were kindly supplied from Ortonuovo Srl (Arbizzano-Santa Maria, VR). The collected samples were cleaned, comminuted, freeze-dried and stored at -20 °C until use. In a second step of the experimental research, lyophilized samples of the microalga *Chlorella vulgaris*, another carotenoid-rich substrate, were tested. All samples were characterized for their water content and water activity (aw).

Natural HDESs were prepared according to the method proposed by Dai et al. (2014), with slight modifications. The two solid components in pre-set molar ratios were placed in a bottle with a stirring bar and cap and heated in a water bath at 70 °C for 30-60 min, till a clear liquid was formed. For the carotenoid extraction from plant by-products, eleven Natural HDESs were prepared utilizing monoterpenes (camphor and thymol) as hydrogen bond acceptors and carboxylic acids (lactic and decanoic acids) as hydrogen bond donors; furthermore DL-menthol was utilized as both HBA and HBD. For the extraction from *Chlorella vulgaris* biomass, other seven natural HDESs were prepared using fatty acids (caprylic, pelargonic, capric acid and lauric acids) as HBAs and HBDs. All natural HDESs were physicochemically characterized assessing their aw, density (γ) and dynamic viscosity (μ). Furthermore, the Natural HDESs’ density was assessed in the temperature range 20 - 60 °C andthe viscosity in the temperature range 20-60 °C and applying different shear-rates, ranging from 50 to 300 s-1.

# Assessment of the extraction efficiency

# Preliminary extraction tests were performed adding 0.1 g of lyophilized sample to 5 mL of each Natural HDES (sample:solvent 1:50). The mixture was vortexed at 25 °C for 60 s and then kept under continuous mixing for 30 min using a rotating mixer Afterwards, the sample was sonicated for 60 min at 45 kHz, before being centrifuged at 3900 RCF for 10 min. The amount of carotenoids extracted was assessed by spectrophotometric measurement, taking readings at 450 nm, as reported by Scott (2001).

# For the extraction using plant by-products as substrates, a given aliquot of the supernatant was diluted with acetone (1:5) taking the absorbance at 450 nm and the carotenoid content was quantified as β-carotene, while, for the microalga matrix, a higher dilution with acetone (1:50) was utilized. The extracting trials were performed in triplicate. As a reference, the extractions were carried out also with acetone, an organic solvent that finds use in the recovery of carotenoids from vegetable matrices for food purposes. Statistical analysis of the data was performed using the software XLSTAT Premium (Version 2020.3.1, Addinsoft, Paris, France). Data were analyzed by one-way ANOVA and significant differences among means were computed by Tukey’s HSD test (Honestly Significantly Different) at a significance level of 0.05.

# Optimization of the extraction processes

# The selection of the best performing Natural HDESs for the extraction from the four plant by-products was achieved based on the results of the preliminary extraction tests. Afterward, the optimum conditions for maximizing the extraction efficiency were evaluated by implementing a three-factor, three-level Box–Behnken experimental design (BBD) combined with response surface modeling (RSM). For this study, the effect of HBD:HBA molar ratio (x1), solvent to sample ratio (x2) and extraction time (min, x3) were selected as independent variables and studied at three different levels coded as -1, 0 and +, with 5 central points, for a total of 17 runs. In order to obtain a more robust data set, each run, except the central ones, was carried out twice, for a total of 29 experiments for each investigated HDES. The response variables selected to be optimized were β-carotene and lutein yields (y1 and y2), which were separated on a, C30 column (4.6 × 250 mm, 5 μm, YMC Inc., Wilmington, NC) using a HPLC–diode array detector system. Peaks were separated by gradient elution according to the procedure described by Stupar et al., (2021). With regard to the selected best extracting Natural HDES for the extraction from *Chlorella vulgaris* substrate, the process optimization using the same design of experiment is ongoing. The statistical analysis was carried out using the software Design Expert software (Version 8.0.7.1, Stat-Ease Inc., USA). The optimized values of the three factors were obtained using the software’s desirably function, with values ranging from 0 (completely undesirable response) and 1 (fully desirable response).

1. **Results and Discussion**

**3.1 Natural HDESs preparation and physicochemical characterization**

# Natural HDESs have been reported to possess efficient extraction capabilities towards carotenoid compounds present in foods (Silva et al., 2019; Stupar et al., 2020). In this research, several HDESs were prepared and tested as valuable solvents for the recovery of carotenoids from different matrices. As showed in Table 2, eighteen Natural HDESs were prepared combining different starting materials in specific molar ratios. Eleven (from HDES 1 to HDES 11) were tested for the extraction of the carotenoid fraction from carrot, yellow and red pepper and pumpkin peels, while seven (from HDES 12 to HDES 18) were used for the extraction using a microalgae, *Chlorella vulgaris*, as the substrate*.* A first selection was made observing the solvent stability during storage after their preparation. Actually, HDES 3, 8 and 11 showed thermal instability with tendency to separate when cooled below 25 °C, giving rise to two layers and requiring subsequent heating for their use as an extraction media. Therefore, they were excluded from the subsequent investigation steps. Furthermore, also HDES 17 showed instability when the room temperature dropped at 20 °C, but, due to the easy restore of the liquid state at already 23 °C, the solvent was investigated with the others taking care of maintaining the working temperature at 25 ± 1 °C.

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| **Table 2.** *Composition and physical characteristics* of the investigated Natural HDESs. |
| **NaturalHDES** | **HBA/HBD** | **Molar ratio** | **Density(g/cm3 at 25 °C)** | **Viscosity\*(mPa·s at 25° C)** |
| HDES 1HDES 2HDES 3 | DL-menthol/lactic acid | 1:11:28:1 | 0.9811.0310.898 | 56.7154.82134.68 |
| HDES 4HDES 5 | DL-menthol/decanoic acid | 1:16.5:3.5 | 0.8940.921 | 35.2431.78 |
| HDES 6HDES 7 | thymol/DL-menthol | 1:11:2 | 0.9350.924 | 37.8654.68 |
| HDES 8HDES 9 | thymol/decanoic acid  | 1:13:2 | n.a.0.919 | n.a.18.86 |
| HDES 10HDES 11 | camphor/decanoic acid | 1:21:1 | 0.931n.a. | 25.58n.a. |
| HDES 12HDES 13HDES 14 | caprylic acid/capric acid | 2:13:14:1 | 0.9000.9010.863 | 9.8419.6428.786 |
| HDES 15 | caprylic acid/lauric acid | 3:1 | 0.901 | 12.59 |
| HDES 16 | pelargonic acid/lauric acid | 3:1 | 0.858 | 15.56 |
| HDES 17 | capric acid/lauric acid | 2:1 | 0.892 | 17.12 |
| HDES 18 | pelargonic acid/capric acid/lauric acid | 3:1:1 | 0.896 | 13.58 |
| \* measured at shear rate 50 s⁻1; n.a. = not assessable. |

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| **Table 3**. *Power law model: n and k values calculated for the different Natural HDESs selected.* |
|  | Natural HDESs tested on vegetable by-products |
|  | HDES 1 | HDES 2 | HDES 4 | HDES 5 | HDES 6 | HDES 7 | HDES 9 | HDES 10 |
| n | 0.70 | 0.73 | 0.45 | 0.68 | 0.67 | 0.75 | 0.70 | 0.66 |
| k | 149.62 | 142.19 | 135.24 | 83.73 | 173.01 | 107.39 | 89.25 | 182.76 |
|  | Natural HDESs tested on microalgae |
|  | HDES 12 | HDES 13 | HDES 14 | HDES 15 | HDES16 | HDES17 | HDES18 |  |
| n | 0.65 | 0.66 | 0.81 | 0.65 | 0.64 | 0.78 | 0.78 |  |
| k | 93.51 | 73.82 | 8.15 | 210.92 | 306.79 | 84.76 | 46.32 |  |
| n = power law index; k = consistency index |

The density and the viscosity values assessed for the various solvents prepared are reported in Table 2. Density ranged from 0.858 to 1.031 g/cm3 at 25 °C, with HDES 16 and HDES 2 showing the lowest and the highest density values, respectively. As far as viscosity, the obtained values showed how all the Natural HDESs, except HDES 3 (excluded for its instability), fulfil one of the four standards established to assess the sustainability of these solvents from a chemical engineering point of view, namely a viscosity smaller than 100 mPa·s (van Osch et al., 2020). Furthermore, as reported in the previous section, the Natural HDES viscosity was assessed in the shear rate range 50 – 300 s-1. This investigation was carried out in order to acquire information on the rheological flow behavior of these solvents and the data obtained show that the eighteen HDESs are non-Newtonian fluids. In particular, they have a shear-thinning behavior, with a decrease in the viscosity when higher values of shear rate are applied. This assumption was substantiated by the “n” values obtained when using the Power Law Model (Table 3).

# 3.2 Assessment of the extraction efficiency

# Of the initial 18 natural HDES prepared, 8 were tested for the extraction of carotenoids from vegetable peels (HDES 1-2, 4-7, 9-10) while the remaining 7 were tested for the extraction of the compounds of interest from *Chlorella vulgaris* (HDES 12-18). Their composition is reported in Table 1. The extraction recoveries, calculated as percentage of the extraction yield obtained by using acetone, were statistically evaluated by using ANOVA in order to assess differences and to identify the solvent(s) suitable to be further investigated (Figure 1).

**Figure 1.** *Percentage of carotenoid recovery from different matrices using different Natural HDESs with reference to acetone extraction. For each matrix, values with different letters are significantly different for p<0.05.*

# As far as the four different vegetable matrices, the HDESs utilized allow to achieve recoveries higher than 80% for 2 out of 4 of the tested substrates, with values near or over 100% when working on yellow and red pepper. In the case of the carrot, the recovery was still high, but only around 80% of the potentially allowable carotenoids were extracted using HDES 6 and 7. Generally speaking HDES 6 and 7 allowed to obtain the best results, except when working with pumpkin skin, In this case the highest recovery was obtained by using HDES 2, about 95%, while for all the other DES the percentage was very limited, not reaching the 20% value. Surprisingly, the HDES 7 showed the worst performance, with just a 9% recovery. Actually, HDES 6 and 7 were both prepared with thymol/DL-menthol at different molar ratios, and the increase of the DL-menthol amount, going from 1:1 to 1:2 as molar ratio negatively influenced the extraction percentages. Taking into account the above reported results, the HDES 6 was chosen to be further investigated when working with carrots and yellow pepper, while HDES 2 was chosen to be tested working on pumpkin skins. For red pepper by-products, the choice was oriented to the use of the HDES 9, because even if the assessed recovery was slightly less than that obtained with HDES 6 (106 % *vs* 109%) the data were not significantly different at p<0.05. Furthermore, working with a DES made up with thymol/decanoic acid rather than thymol/DL-menthol was considered a potentially positive aspect due to the lower cost of the solvent and its less intense menthol aroma.

# With regard to the extraction from the microalga, very encouraging results were achieved, since the extraction efficiency ranged from 114 to 227 % with respect to the acetone extraction at the same operating conditions. The ANOVA analysis allowed identifying significant differences among the various solvents (Figure 1), with HDES 12, 14 and 16 giving the highest recoveries. The worst extraction performances were recorded when using capric acid and lauric acid as HDES constituents (HDES 17 and 18). On the basis of the obtained results, and being HDES 12, 13 and 14 realized with the same components, but at a different molar ratio, it was decided to continue the investigation optimizing the extraction process with reference to the composition of the HDES 16 and on the use of caprylic acid/capric acid as the HDES components.

# 3.3 Optimization of the extraction processes

# In order to identify the best extraction conditions, a BBD was utilised as previously described. In Table 4 are reported the values of the independent variables utilised to optimise the extraction process. In total 116 experiments were carried out and the resulting data were utilised to optimise the recovery of the carotenoid fraction from each substrate. The estimated optimal extraction conditions were as follows: solvent/sample ratio 50 for all the substrates; HBA/HBD ratio 2.5:1 for carrots, 5.75:1 for yellow pepper, 1.95:1 for red pepper, 4.68:1 for pumpkin skins; extraction time 30 min for carrots and red pepper, 76 min for yellow pepper, and 90 min for pumpkin skins. Practical validation of the model is actually undergoing, as well as the extraction optimisation of the carotenoid fraction from *Chlorella vulgaris* with the selected Natural HDESs.

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| **Table 4.** *Natural HDES Extraction optimization: values of the independent variables for recovering the carotenoid fraction from vegetable by-products.* |
| **Independent variables** | **Levels** |
| (-1) | (0) | (1) |
| HBA:HBD molar ratio |  |  |  |
| *HDES 6* (carrot and yellow pepper) | 0.25 | 4 | 7.75 |
| *HDES 9* (red pepper) | 0.5 | 1.50 | 2.50 |
| *HDES 2* (pumpkin skin) | 0.25 | 3 | 5.75 |
| Solvent-to-sample ratio | 10 | 30 | 50 |
| Extraction time | 30 | 60 | 90 |

1. **Conclusions and future perspectives**

# The aim of this PhD project is the optimization of a green extraction technique using Natural HDESs for the recovery of carotenoids from by-products of the industrial processing of vegetable foods, as well as from an unconventional food source, the microalga *Chlorella vulgaris*. The initial activities were carried out using the scientific approach of carefully reviewing the pertinent literature in the field of DESs, to identify a promising research gap to work on.

# Nowadays, the use of Natural HDESs as solvents for the extraction of apolar molecules from food matrices has been investigated only in a limited number of papers, while the expansion of the knowledge in this area is of noticeable interest. The research involved the study of the physicochemical characteristics of 18 Natural HDESs as well as their use at preset conditions to identify the most suitable ones for the carotenoid extraction. Out the 18 solvents, 3 were selected for further investigation on real industrial substrates and 2 for the microalgae. The extraction process was studied and optimized for the vegetable by-products, while for microalgae this part of the research is undergoing, ending this PhD project in March 2024. In the meantime, recovery and recycling of the Natural HDESs will be investigated, as well as the possibility to incorporate the extracts in food supplements and/or cosmetic products, carrying out the study in cooperation with companies of the field, which have shown interest in the research.

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