UNIVERSITÀ DEGLI STUDI DELLA BASILICATA
SAFE - Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali

Università degli Studi della Basilicata

PhD in

Agricultural, Forest, and Food Sciences

TITLE

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

Scientific Area (SSD)
AGR/15 - Scienze e Tecnologie Alimentari

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Cycle XXXVI

A tutte le persone che mi hanno sostenuto in questo percorso

ABSTRACT

Carotenoids are a group of pigments recognized for their antioxidant properties and preventive effects on developing many diseases. The global market of these pigments is growing constantly and is expected to reach US$ 2 billion by 2031 due to the rising interest in healthy and natural foods accompanied by the increase in dietary supplement consumption. Thus, the growing demand for naturally sourced carotenoids, now representing much less than half of the total offer, is an emerging issue, considering the production costs and the environmental sustainability of the processes. In this regard, the growing interest in "*green chemistry*" and the increasing demand for sustainable processes have sparked the scientific community's attention. The researchers are more and more focusing on infocused on investigating Natural Hydrophobic Deep Eutectic Solvents (NaHDESs) and their potential applicationse generally accepted as environmentally friendly. These green solvents are generally formed by mixing at least two components, a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD), at a given molar ratio, that, by establishing hydrogen bonds, cause a significant melting point depression of the mixture and a phase transition, from solid to liquid. Furthermore, for NaHDESs preparation, only moieties derived from natural sources can be used, and the obtained mixtures should have a resultant hydrophobicity. The physicochemical properties and the extraction capability towards different molecules result from the specific combination of several starting materials and the selected molar ratios. Monoterpenes, carboxylic, and fatty acids are the most common natural HBAs and HBDs utilized for the NaHDESs formation which results to be characterized by a very low cost and negligible ecological impact and toxicity. Additionally, their high biocompatibility and food-grade nature open the way for new direct applications of the extracts "*as such"* in the food, cosmetic, and pharmaceutical industries.

On these bases, this PhD project aimed to develop and optimize green extraction processes using different NaHDESs to recover carotenoids from vegetable products and by-products derived from the food processing industry, considering both process sustainability and productiveness aspects.
The PhD activities carried out to achieve the goal of this PhD project were:

- Implementation of green extraction processes through the selection of suitable NaHDESs and rich-carotenoid matrices, the assessment of the physicochemical properties of the selected solvents, and the selection of the best-performing ones for each matrix;

- Optimization of the extraction processes by identifying the proper combination between the HBA:HBD molar ratio, the solvent sample ratio, and the optimum extraction time;

- Antioxidant stability characterization of the carotenoid-rich NaHDES extracts and development of food applications.

The PhD project's initial activity was focused on the study of the scientific literature related to the field of Deep Eutectic Solvents. First of all, topics related to the development of this class of solvents, their classification in subcategories, their principal physicochemical characteristics, and their food applications were detailed. Then, a comprehensive review of the use of NaHDESs for extracting biomolecules from foods was realized, focusing on the advantages and weaknesses of the proposed extraction techniques, taking into account both the extraction efficiency and the scalability of the process **(Chapter 1).**

The analysis of the literature suggested the great potential of NaHDESs for effectively extracting carotenoids from foods and food by-products matrices, and also highlighted how more in-depth investigations are still needed to optimize the extraction process. The first adopted approach was to focus the attention on carotenoid-rich by-products derived from the processing of fresh vegetables since the implementation of a circular economy model could represent an added plus of the green extraction process. Therefore, the peels of fresh carrots, red and yellow peppers, and pumpkins were selected as extraction substrates. Eleven NaHDESs, based on terpenes and carboxylic acids, were investigated for their physicochemical properties and their extraction efficiency for the carotenoid recovery, with reference to that obtained when using acetone as a solvent. The best performing NaHDES for each substrate was selected based on the stability during storage and the results of preliminary extraction tests carried out at preset operating conditions. Afterward, a Box Behnken Design (BBD) was utilized to optimize the HBA:HBD molar ratio of the selected NaHDESs, the solvent sample ratio, and the extraction time to maximize the carotenoid yields (**Chapter 2**).

In the second step of the experimental research, seven other NaHDESs, based on fatty acids, were investigated to extract carotenoids from an emerging food matrix, the microalga *Chlorella vulgaris*. The research design adopted was almost unmodified with respect to that previously described. The initial screening of the most suitable solvent and the subsequent optimization step were improved taking into account also the antioxidant properties of the obtained extracts (**Chapter 3**).

Through the above-reported activities, five carotenoid-rich NaHDES extracts were obtained by performing the extraction processes in the identified optimized conditions and were characterized for their carotenoid content. The extracts derived from carrot peels and the microalga *Chlorella vulgaris* resulted in having the highest carotenoid content and were selected for the formulation of fortified cocoa and hazelnut spreadable creams. Therefore, the experimental activities were organized as follows (**Chapter 4**):

- Characterization of the extracts, studying the antioxidant stability during storage at different environmental conditions, and comparing the results with the antioxidant stability of extracts obtained at the same extracting operating conditions but using acetone as a solvent;
- Characterization of the four fortified creams obtained by adding two different amounts of each extract to the cream base. The analyses were carried out to evaluate the effect of the fortification in terms of color, textural and rheological properties, carotenoid content, and antioxidant activity with reference to the control formulation.

Based on the results of this PhD project, it can be concluded that the use of NaHDESs as a promising alternative to traditional organic solvents might provide pivotal advantages, with extraction performances comparable or, in some cases, even higher than those attainable with acetone, a GRAS solvent that can be utilized, with some constraints, in producing food grade extracts. Furthermore, the NaHDES food-grade nature may result in possible uses of the extracts *'as such',* thus reducing the processing costs related to the usually required de-solventization steps for recovering the extracted biomolecule.

RIASSUNTO

I carotenoidi sono noti per i loro benefici per la salute e le proprietà antiossidanti. Il mercato globale di questi pigmenti è in costante crescita e si stima che il suo valore raggiungerà i 2 miliardi di dollari entro il 2031. Ciò è dovuto al crescente interesse dei consumatori per un'alimentazione basata su prodotti sani e naturali e al conseguente aumento del consumo di integratori alimentari. Pertanto, l'aumento della domanda di carotenoidi derivanti da fonti di origine naturale, che ora rappresentano molto meno della metà della offerta totale, rappresenta un problema emergente, considerando anche i costi di produzione connessi alla loro produzione e l'aspetto di sostenibilità ambientale dei processi. A tal proposito, la comunità scientifica ha focalizzato le sue risorse allo sviluppo di processi sostenibili, seguendo i principi della "chimica verde" e molte ricerche sono state incentrate allo studio le l potenziali applicazioni di una classe emergente di solventi generalmente riconosciuti come sostenibili per l'ambiente, definiti solventi eutettici profondi idrofobici naturali (NaHDESs). In termini generali, la preparazione di questi solventi verdi prevede la miscelazione di almeno due componenti, un accettore di legame idrogeno (HBA) e un donatore di legame idrogeno (HBD) ad un dato rapporto molare, che, stabilendo legami idrogeno, causano una diminuzione del punto di fusione della miscela e la conseguente transizione di fase, dallo stato solido a quello liquido. Inoltre, per la preparazione dei NaHDES, possono essere miscelate esclusivamente sostanze provenienti da fonti naturali e le risultanti miscele devono essere caratterizzate da idrofobicità. Le proprietà fisico-chimiche, così come le capacità estrattive nei confronti di diverse molecole, sono derivanti dalla combinazione specifica di diverse sostanze di partenza e dei rapporti molari selezionati tra di loro. I monoterpeni, gli acidi carbossilici e gli acidi grassi sono tra le più comuni sostanze utilizzate come HBA e HBD per la formazione dei NaHDESs, che risultano avere quindi un costo limitato e un impatto ecologico e una tossicità trascurabili. Inoltre, la loro elevata biocompatibilità e la loro compatibilità alimentare consentono di progettare delle applicazioni dirette degli estratti in quanto tali nell'industria alimentare, cosmetica e farmaceutica.

Sulla base di quanto esposto, l'obiettivo del presente progetto di dottorato è stato lo sviluppo e l'ottimizzazione di processi di estrazione verde mediante l'uso di NaHDESs per il recupero di carotenoidi da prodotti e sottoprodotti derivanti dalla lavorazione industriale di alimenti vegetali, tenendo in considerazione gli aspetti di sostenibilità e di produttività dei processi.

Le attività di dottorato svolte per il conseguimento di tale scopo sono state le seguenti:

- Implementazione di processi di estrazione verde attraverso la selezione di NaHDESs idonei e di matrici ricche in carotenoidi, valutando le proprietà fisico-chimiche dei solventi investigati e selezionando quelli più performanti per ciascuna matrice;
- Ottimizzazione dei processi di estrazione identificando la corretta combinazione tra rapporto molare HBA:HBD, rapporto solvente campione e tempo di estrazione ottimale;
- Caratterizzazione della stabilità antiossidante degli estratti NaHDES ricchi di carotenoidi e sviluppo di applicazioni alimentari.

Le prime attività del progetto di dottorato sono state focalizzate sullo studio della letteratura scientifica nel campo dei Solventi Eutettici Profondi, investigando e approfondendo gli aspetti relativi allo sviluppo di questa classe di solventi, la loro classificazione in sottocategorie, nonché le loro principali caratteristiche fisico-chimiche e le numerose applicazioni alimentari. È stata poi redatta una revisione della letteratura esaustiva sull'uso di NaHDESs per l'estrazione di biomolecole dagli alimenti, mettendo in evidenza i vantaggi e i le lacune delle tecniche di estrazione proposte, considerando l'efficienza di estrazione e la scalabilità del processo **(Capitolo 1).**

Mediante l'analisi della letteratura si è evidenziato il grande potenziale dei NaHDESs per l'estrazione dei carotenoidi dagli alimenti e dai sottoprodotti alimentari. Allo stesso tempo, le limitazioni ancora riscontrate in questo campo di applicazione hanno messo in luce la necessità di indagini più approfondite per l'ottimizzazione del processo.

Il primo approccio adoperato è stato quello di focalizzare l'attenzione sui sottoprodotti della lavorazione di vegetali ricchi in carotenoidi, poiché l'implementazione di un modello di economia circolare potrebbe rappresentare un ulteriore valore aggiunto del processo di estrazione verde. Pertanto, le bucce di carota, di peperone rosso e giallo e di zucca sono state utilizzate come substrati di estrazione. La caratterizzazione fisico-chimiche di undici NaHDESs, costituiti da terpeni e acidi carbossilici, è stata condotta e successivamente ne è stata testata l'efficienza di estrazione comparandola ai risultati ottenuti usando l'acetone come solvente. La selezione del NaHDES più performante per ciascun substrato è stata ottenuta considerando la stabilità durante la conservazione e i risultati dei test di estrazione, condotti mantenendo fissi i parametri di processo. Successivamente, è stato eseguito un Box Behnken Design per ottimizzare il rapporto molare HBA:HBD dei NaHDESs selezionati, il rapporto campione:solvente e il tempo di estrazione per massimizzare la resa dei carotenoidi **(Capitolo 2)**.

In una fase successiva dell'indagine, altri sette NaHDESs, costituiti da acidi grassi, sono stati testati per l'estrazione di carotenoidi da una matrice alimentare emergente, la microalga *Chlorella vulgaris*, mantenendo pressoché invariato l'approccio sperimentale e migliorando gli aspetti relativi alla valutazione dell'efficienza di estrazione. Il disegno sperimentale adottato è rimasto pressoché invariato rispetto a quello precedentemente descritto. Lo screening iniziale e la successiva fase di ottimizzazione sono stati perfezionati con un maggior numero di analisi sull'efficienza estrattiva (**Capitolo 3**).

Mediante le attività precedentemente descritte, cinque estratti di NaHDES ricchi di carotenoidi sono stati ottenuti eseguendo i processi di estrazione nelle condizioni ottimizzate e sono stati caratterizzati per il loro contenuto in carotenoidi. Gli estratti derivanti dalle bucce di carota e dalla microalga *Chlorella vulgaris* sono risultati essere quelli più ricchi di carotenoidi e sono stati selezionati per la formulazione di creme spalmabili fortificate al cioccolato. Pertanto, le attività sperimentali sono state organizzate come segue (**Capitolo 4**):

- Caratterizzazione degli estratti, studiando la stabilità antiossidante durante la conservazione in diverse condizioni ambientali, e confrontando i risultati con la stabilità antiossidante degli estratti ottenuti nelle stesse condizioni di estrazione, ma utilizzando acetone come solvente;
- Caratterizzazione delle quattro creme fortificate, ottenute aggiungendo alla crema base due quantità diverse di ogni estratto. Le analisi sono state effettuate per valutare l'effetto della fortificazione in termini di colore, proprietà reologiche, contenuto di carotenoidi e attività antiossidante rispetto alla crema controllo. Inoltre, ulteriori approfondimenti scientifici saranno fondamentali per indagare l'accettabilità da parte del consumatore.

Sulla base dei risultati di questo progetto di dottorato, si può concludere che l'uso di NaHDESs come alternative promettenti ai solventi organici tradizionali offre vantaggi fondamentali, con efficienze estrattive comparabili o, in alcuni casi, anche superiori a quelle ottenute con solventi convenzionali. Inoltre, la compatibilità alimentare di questi solventi consente possibili applicazioni degli estratti in quanto tali, evitando così la fase di recupero delle biomolecole estratte e riducendo i costi del processo di estrazione.

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1. Hydrophobic Deep Eutectic Solvents in the food sector: focus on their use for the extraction of bioactive compounds

This chapter is a state-of-the-art chapter, and its content has been already published:

Sportiello, L., Favati, F., Condelli, N., di Cairano, M., Caruso, M.C., Simonato, B., Tolve, R., & Galgano, F. (2023). Hydrophobic deep eutectic solvents in the food sector: Focus on their use for the extraction of bioactive compounds. *Food Chemistry*, 405, 134703.

1.1. Introduction

Consumers are increasingly focusing their eating habits on healthier foods, driving more and more the food industry towards the production of functional food items and dietary supplements. Within this frame, the use of bioactive molecules is gaining attention and generating the need for investigating new sources as well as innovative extraction processes. Furthermore, also pharmaceutical and cosmetic industries have an interest in new moieties, which may be utilized in product formulation. Nowadays, the industrial recovery of these moieties represents a challenging step, with problems related to costs, efficiency, selectivity and environmental sustainability (Choi and Verpoorte, 2019). With reference to the latter issue, the concept of "Green Chemistry" as "the design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances" has been formulated more than 30 years ago and, Anastas & Warner (1998) presented twelve principles as a set of criteria and guidelines for developing sustainable processes. In particular, with principles number 5 and 10 the authors highlighted the requirement of using safer solvents, which should be harmless as well designed to break down into innocuous degradation products not persisting in the environment. Within this frame, in the last 20 years, a growing number of research papers have been published dealing with Deep Eutectic Solvents (DESs) as a potential replacement for the traditional ones, with the research of Abbott et al. (2003) being a milestone in this research area. Initially, in the search for alternative green extraction procedures, the use of Ionic Liquids (ILs) was proposed. ILs are defined as salts deriving from the combination of an organic cation and an anion, characterized by a melting point below 100 ◦C, being in most cases liquids at room temperature (van Osch et al., 2017). The original concept of this class of solvents was based on non-toxicity, non-volatility, non-flammability, and stability in air and water. Actually, the majority of cations and anions combinations used for ILS preparation allowed for the attribution of green

properties for ILs. However, throughout the years several questions have raised about their toxicity towards living organisms (Flieger & Flieger, 2020) and, in general, their green nature. In this scenario, DESs have been considered as a potential substitute to ILs. While several attempts have been made, a unique DES definition is difficult to find (Zhang et al., 2012; Francisco et al., 2013; Smith et al., 2014). The most accepted one defines DESs as "mixtures of two or more pure compounds for which the eutectic point temperature is below that of an ideal liquid mixture, presenting significant negative deviations from ideality" (Martins et al., 2018). Although ILs and DESs share several features and properties, they represent independent groups of solvents, being the preparation method and the chemical nature of the components the most important differences. Regarding the starting materials, organic heterocyclic cations and organic or inorganic anions represent the base for ILs production, whereas DESs are prepared by mixing hydrogen bond acceptors (HBAs) with hydrogen bond donors (HBDs). Furthermore, the synthesis of ILs often involves several steps and requires different reagents, thus generating disposal problems of the resultant by-products and wastes. In comparison with ILSs, DESs often show some interesting characteristics, such as easier preparation, lower production cost, limited hazardous nature and higher biodegradability and stability (Plotka-Wasylka et al., 2020) (Table 1). However, as far as biodegradability and toxicity, it must be pointed out that, according to the nature of the moieties utilized for preparing the different DESs, the impact of these solvents might not be considered always negligible, so they should rather be considered a class of supposedly environmentally friendly solvents. While much research has been carried out on hydrophilic DESs, only a limited number of papers have focused attention on hydrophobic DESs (HDESs). In this context, the attempt to carry out an in-depth literature review on HDESs could be useful to pave the way for future developments. Through a thorough scrutiny of the most pertinent and recent articles, this review reports the available information on HDESs' definition and physicochemical properties and discusses with a critical approach the results related to the use of HDESs as alternative solvents for recovering biomolecules from foods and plants matrices.

Table 1.1. Main characteristics of several Ionic Liquids and Deep Eutectic solvents.

Ionic Liquids (ILs)	Deep Eutectic Solvents (DESs)
Organic heterocyclic cations and organic or inorganic anions as starting materials	Hydrogen bond acceptors (HBAs) with hydrogen bond donors (HBDs) as starting materials
Ionic bonds interactions	Hydrogen bond interactions
Expensive and problematic to synthetize on a large scale	Quick and easy preparation
Hazardous	Less or no hazardous
Non-biodegradable	Biodegradable

1.2. DES - classification and preparation

DESs, according to their definition (Martins et al., 2018), are formed by mixing a HBA with a HBD compound at a given molar ratio, with a resultant melting point depression of the obtained mixture. The formula Cat^+X^-zY can be used to describe DESs, being Cat^+ any sulfonium, phosphonium, or ammonium cation, X a Lewis base, generally a halide anion, and z the number of Y molecules interacting with the anion. According to Smith et al. (2014), DESs can be classified in four different groups, and their general formulas are reported in Table 1.2.

Table 1.2. General formulas for DESs classification (Smith et al., 2014).

Type	General formula	Terms
Type I	$Cat^+X^-zMCl_x$	M = Zn, Sn, Fe, Al, Ga, In
Type II	$Cat^+X^-zMCl_x \cdot yH_2O$	M = Cr, Co, Cu, Ni, Fe
Type III	Cat^+X^-zRZ	Z = $CONH_2$, COOH, OH
Type IV	$MCl_x + RZ = MCl_{x-1}^{+} \cdot RZ + MCl_{x+1}^{-}$	M = Al, Zn and Z = $CONH_2$, OH

Cat^+ = any ammonium, phosphonium, or sulfonium cation; X = Lewis base, usually a halide anion; z = number of Y molecules interacting with the anion.

Type III eutectics, obtained by using choline chloride as HBA and several different HBDs (e.g. alcohols, amides and carboxylic acids) have sparked interest because of their capability to solvate a broad spectrum of transition metal species, as well as oxides and chlorides. These DESs can be easily prepared, are rather unreactive with water, much of them are biodegradable and their preparation is quite economical. Type III is currently the focus of much research. Choline

chloride is the most widely used quaternary ammonium salt (QAS) for the preparation of these solvents due to its low cost, high availability, biocompatibility and low toxicity. Furthermore, the chloride anion can easily interact with various proton donors via hydrogen bonding. The physicochemical and thermal properties of DESs can be modified by varying the chemical species constituting the solvent. As already mentioned, most of the investigated DESs are hydrophilic (Ruß et al., 2012; Francisco et al., 2013; Smith et al., 2014). Type III DESs are classified into natural deep eutectic solvents (NADESs), low transition temperature mixtures (LTTM), carboxylic acid-based DESs and deep therapeutic eutectic solvents (THEDESs) (Abbott et al., 2004; Florindo et al., 2014; Aroso et al., 2015; Aroso et al., 2016; Yiin et al., 2016). Recently, a new DESs generation has been investigated, Hydrophobic DESs, which can be obtained using components having no or little water-solubility, and can be utilized to extract lipophilic moieties. As HBA ionic or non-ionic compounds can be utilized (e.g. tetralkyl quaternary ammonium/phosphorous salts or monoterpenes), while phenols, carboxylic acids, alcohols and glycols can be used as HBD. However, it should be pointed out that some of the latter may act both as donors or as hydrogen bond acceptors. A distinction between synthetic and natural HDES can be made taking into account the use of potentially non-harmful natural moieties, and thus represent a major issue when dealing with the extraction of molecules that may find use in the food, the cosmetic and the pharmaceutical industry.

As far as DES preparation, this is basically a simple process requiring mixing of the HBA and HBD constituents. Although some HDESs have been easily prepared by mixing at room temperature (Rajabi et al., 2018), the first and most common approach for preparing DESs (by heating and stirring) consists of combining two or more components at a relatively high temperature until a homogeneous fluid is obtained (Gan et al., 2016; Tang et al., 2017). Usually, the reaction temperature is fine-tuned, between 50 and 100 °C, to suit several reaction materials (Florindo et al., 2014). However, when working at high temperatures HCl can be produced and specific interactions between the proton and the carbon in carboxylic and glutaric acids might occur. Furthermore, carboxylic acid can react with a component containing hydroxyl groups giving rise to esters formation (Tang et al, 2021). Taking into account these problems, Florindo et al. (2014) have proposed a DESs preparation method based on grinding of the components, thus obtaining higher purity DESs. Moreover, the heating and stirring method involves the

presence of water, and to overcome the issue, Dai et al. (2013) and Gutierrez et al. (2009) developed two other preparation methods involving evaporation or lyophilization steps.

1.3. HDES ionic and non-ionic

The focus on HDESs is relatively recent and only in 2015 the first papers dealing with HDESs appeared in the literature. In the study carried out by van Osch et al. (2015), the authors combined several QASs with decanoic acid for the extraction of water-insoluble volatile organic compounds and obtained encouraging results regarding yield and efficiency of the developed system. A few months later in the same year, Ribeiro et al. (2015) used other components, DL-menthol and natural acids, for preparing HDESs tested for the extraction of caffeine, isophthalic acid, tryptophan, and vanillin. However, these initial attempts were characterized by some solvent drawbacks, consisting mainly in high viscosity and a tendency to leach in the aqueous phase. In the following year, few works appeared, focused on the development of HDESs for metals removal in aqueous environments (Tereshatov et al., 2016; van Osch et al., 2016). Conversely, 2017 was a year of remarkable growth for this sector, with the development of several interesting applications, namely the recovery of artemisinin from leaves of Artemisia annua for malaria treatment (Cao et al., 2017a), the first micro-extraction for the detection of benzoylurea residuals in water samples (Yang et al., 2017), the use of DES as materials platform for photon upconversion, (Murakami et al., 2017) and the use of HDESs as additives in membranes (Dietz et al., 2017). In the following years, additional papers were published dealing with theoretical approaches (Kollau et al., 2018), the determination of the liquid phase behavior (van den Bruinhorst et al., 2018) and the development of type V DESs. In particular, regarding the latter Abranches et al. (2019) have reported that thymol-menthol mixtures show a non–ideal behavior, resulting from a much stronger hydrogen bond established between thymol and menthol than any present in the pure liquid components. Furthermore, this deviation from ideality was found not to be specific to the thymol-menthol mixture. Studying other compounds, a general behavior was identified and type V DESs were defined as those resulting from adding a substance possessing a hydroxyl group directly linked to an aromatic ring, such as phenolic compounds, to another moiety acting as hydrogen bond acceptor, even if the latter is able to establish hydrogen bonds with itself.

In Figure 1.1. are reported some of the HBAs and HBDs used for HDESs preparation. The main difference between the components of hydrophilic and hydrophobic DESs is the presence

in the latter of long alkyl chains or cycloalkyl groups, hence decreasing the effect of hydrophilic domains (charged with salts) and hydrophilic groups (Florindo et al., 2019). Van Osch et al. (2019) presented four chemical criteria to evaluate the sustainability of HDESs taking into account: a) viscosity (<100 mPa·s); b) density (being 50 kgm^{-3} the minimum needed difference between HDESs and water when mixed); c) limited HDES transfer to the water phase and d) small or negligible pH change. Moreover, among the reported HDESs, the main distinction leads to a categorization into two types: ionic and non-ionic HDESs, based on whether or not an ionic component is present.

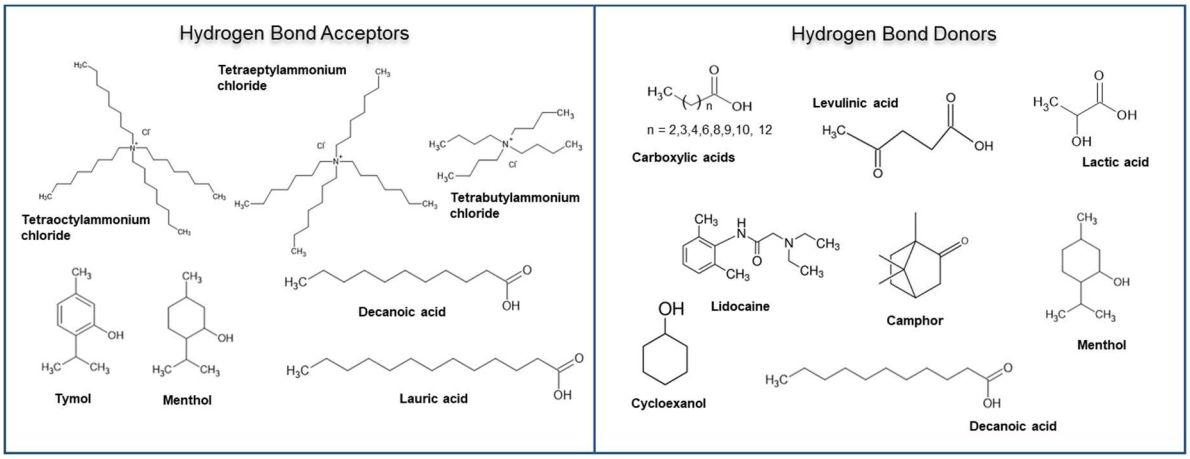

Figure 1.1. HBAs and HBDs used for HDESs synthetization.

The first ionic HDESs were obtained mixing in different amounts a long-chain QAS and decanoic acid (van Osch et al., 2015). The observed high hydrophobicity of the resultant HDESs could be ascribed to the low water content and the low QAS leaching rate.

In order to optimize the hydrophobicity characteristics, subsequent studies investigated the effect of using QAS having different alkyl chain lengths or using long-chain and unsaturated alcohols with carboxylic and hydroxyl groups (van Osch et al., 2015; Tereshatov et al., 2016; Tang et al., 2018; Phelps et al., 2018; Liu et al., 2019a; Dwamena & Raynie, 2020). It should be highlighted how several saturated and unsaturated alcohols (e.g. butanol, hexanol, octanol, 1-decanol, cyclohexanol, DL-menthol, etc.) besides being used as HBD, could also act as HBA to form HDESs when combined with methyl trioctyl ammonium chloride (Cao et al., 2017a; Tang et al., 2018).

Other studies have pointed out the possibility to produce HDESs using non-ionic components, characterized by the presence of polar groups in both the HBA and HBD moieties. The first

solvents of this type that were proposed had DL-menthol as HBA species and various short-chain acids (acetic, lactic and pyruvic acid) as HBD components (Ribeiro et al., 2015). Others non-ionic HDESs were developed combining menthol with carboxylic acids having a chain length variable from C1 up to C12 (Ribeiro et al., 2015; Florindo et al., 2017). Subsequently, some research groups have utilized a number of terpenoid-based compounds (e.g. borneol, camphor, sobrerol and thymol) to prepare new forms of HDESs (Makoś et al., 2018; Martins et al., 2019). In the recent years, several new non-ionic HDESs were formed with uncommon compounds: van Osch et al. (2016) experimented the combination of pain relievers (e.g. lidocaine and atropine) and fatty acids; van den Bruinhorst et al., in 2019 reported other HDES types based on trioctylphosphine oxide and Florindo et al. (2018a) developed low viscosity DESs exclusively consisting of fatty acids.

Nowadays, much research is devoted to investigating the use of food grade moieties for HDESs preparation and their potentialities in various areas (Table 1.3.).

1.4. HDES physicochemical properties

Organic solvents are generally used in liquid-liquid and solid-liquid extractions as extracting media, presenting a series of considerable limitations. The main drawbacks are related to their toxicity and non-biodegradability, with negative consequences on the environment. Furthermore, when the integrity of the target substances is pivotal for their use, the extraction with organic solvents can be pointless as they can cause degradation of the extracted compounds. On this basis, the applicability of a class of solvents such as DESs can be a great chance to overcome these issues, as long as they are able to provide an extraction efficiency comparable to that of common solvents, or at least acceptable taking into account the inherent environmental issues. Reasonably, the extraction capacity is depending not only on the interaction with the substrate and the target molecules, but also on the main HDESs physicochemical properties.

Table 1.3. List of some of HDESs prepared by using food grade components

HBA	HBD	Molar ratio	Preparation	References
(-)-menthol	OctA DecA DoDecA	0.55:0.45 0.65:0.35 0.75:0.25	heating and stirring at 250 rpm at 42 °C	Hümmer et al., 2018
choline chloride	phenethyl alcohol	1:4	stirring at room temperature	Rajabi et al., 2018
DL-menthol	DoDecA	0.5:1, 1:1,1.5:1, 2:1, 2.5:1	reflux condensing for 1 h at 50 °C with stirring	Verma et al., 2018
L-menthol thymol carvacrol	levulinic acid	1:1, 3:2, 2:3 1:1, 3:2, 2:3 1:1, 3:2, 2:3	heating and stirring at 45 °C for 20-30 min	Bezold & Minceva, 2018
DL-menthol	acetic acid lactic acid lauric acid pyruvic acid	1:1 1:2 2:1 1:2	heating at 50°C for 15 min	Ribeiro et al., 2015; Silva et al., 2019
DL-menthol	acetic acid butyric acid DecA DoDecA hexanoic acid levulinic acid OctA pyruvic acid	1:1 1:1 1:1 2:1 1:1 1:1 2:1 1:2	heating and stirring at 80°C	Florindo et al., 2017; Stupar et al., 2021
lauric acid	OctA nonanoic acid DecA	1:3 1:3 1:2	heating at 40°C until a homogeneous clear solution	Florindo et al., 2018
thymol	camphor DecA	7:3, 3:2, 1:1 3:2, 1:1, 1:2, 1:3	heating and stirring at 60 °C	Makoś et al., 2018
L-menthol	borneol camphor sobrerol	7:3 1:1 9,5:0,05	heating and stirring for 30 min	Martins et al., 2019
thymol	borneol sobrerol	1:1 1:1	heating and stirring for 30 min	Martins et al., 2019
camphor	sobrerol	7:3	heating and stirring for 30 min	Martins et al., 2019
norneol	sobrerol	6:4	heating and stirring for 30 min	Martins et al., 2019
thymol	coumarin	1:1, 2:1	stirring at room temperature	Van Osch et al., 2019

DecA = decanoic acid.; DoDecA = dodecanoic acid; OctA= octanoic acid.

1.4.1. Density

Among the DESs' physicochemical characteristics, density is of utmost relevance being a function of the molecular packaging and of the interaction strength between the constituents. Therefore, it is a pivotal parameter to be taken into account when assessing the DES suitability for use as an extracting media. In the literature, the reported HDESs density ranges from 0.85 to 1.5 g·cm-3, but often is similar to that of water. However, if both HDES components show higher density than water, the resultant density will be higher than that of water or vice versa (van Osch et al., 2020; Cao & Su, 2021; Zainal-Abidin et al., 2021).

The HBA and HBD nature can further affect the resultant HDES density that, according to the HDB component, can even decrease with an increasing length of the HBA alkyl chain. For instance, the density of ammonium-based DESs in molar ratio 1:2 follows the order: tetrabutyl ammonium chloride/decanoic acid> tetraeptyl ammonium chloride/decanoic acid> tetraoctyl ammonium chloride/decanoic acid (van Osch et al., 2019). However, Martins et al. (2018) have reported that when preparing thymol-based HDESs by adding acids, a density decrease can be observed when the acid chain length decreases. Furthermore, data in the literature show that a linear decrease in HDES density occurs when increasing the temperature (Ribeiro et al., 2015).

1.4.2. Viscosity

Among the various physical characteristics that directly influence the possible use of HDESs, viscosity is one of the most important, affecting important extraction parameters such as internal and external mass transfer coefficients of the utmost importance in recovering moieties of interest from solid substrates. Therefore, a low viscosity is desirable, especially when an industrial scale-up is expected. HDESs show a wide range of values (7-86,800 mPa•s) theoretically allowing a fine-tuning of the extraction process (van Osch et al., 2020; Cao & Su, 2021), and between the two types of hydrophobic DES, the non-ionic ones show lower viscosities (<219 mPa•s) (Tang et al., 2021). From a chemical viewpoint, high viscosity values might be expected for HDES, because they are formed through hydrogen bonding interactions that in turn could reduce the mobility of the molecules. However, a definite rule allowing the prediction of HDES viscosity cannot yet be defined, even when using a given HBA with various HBDs at set molar ratios. Viscosity can depend on the HBA nature and Tang et al. (2021) have reported as an example that the following HDES viscosity order can be observed for some HBA components: QASs > menthol > thymol> long chain fatty acid. The high viscosity recorded when using QASs could

be ascribed to coulombic forces correlated to the salt nature. As far as the working temperature, viscosity is definitely affected by temperature in an inverse relationship, because the energy supplied by a thermal increase may affect the electrostatic links between DES components, causing their break up.

1.4.3. Melting point and degradation temperature

One of the key points in using DESs is to know the thermal range of their applicability, which is the range delimited by the lower temperature at which DESs are in the liquid state and the upper limit represented by the temperature at which DES degradation occurs. The first can be defined by the melting point (MP), which for a DES is lower than that exhibited by every single component. According to the data reported in the literature, HDESs melting point is below ambient temperature and, in some cases, two different melting points have been reported for the same HDES. This is the case of HDESs obtained using DL-menthol and this particular behavior can be explained taking into account the fact that DL-menthol has at least two polymorphs α and β, which could be accountable for the observed discrepancy (Ribeiro et al., 2015; van Osch et al., 2020). The strength of the interactions between the HDES constituents and the alkyl chain structure has an important impact on the solvent melting point (Abbott et al., 2004; van Osch et al., 2015). In general, for ionic and non-ionic HDES, the MP value is a direct function of the fatty acid alkyl chain length. Furthermore, when dealing with ionic HDESs, their MP value is influenced by the presence of both anions and cations present in the HBA.
Ultimately, the melting point value must be assessed taking into account the entire solid-liquid phase behavior, in order to identify the true eutectic point. As far as the degradation temperature, the chemical nature of the HDES constituents significantly affects this parameter and the literature data report values between 95 and 267 °C, being in general slightly lower than the values exhibited by ILs and hydrophilic DESs (Cao & Su, 2021). As a rule of thumb, ionic HDESs show higher thermal stability, while for non-ionic HDESs an increase in temperature above a specific limit can cause evaporation or sublimation, rather than simple degradation (van Osch et al., 2019). Recently, Dietz et al. (2019) have investigated the total vapor pressure of six HDESs [decanoic acid/lidocaine (4: 1), decanoic acid/lidocaine (3:1), decanoic acid/lidocaine (2:1), decanoic acid/menthol (1:1), decanoic acid/thymol (1:1) and thymol/lidocaine (2:1)] reporting how these solvents show very low vapor pressures in comparison to those of organic solvents commonly utilized in extraction processes. For example, when working in a wide

temperature range (47-107 °C) the reported volatility of the decanoic acid/menthol HDES showed to be 150-1000 times lower than that of toluene. Furthermore, it is important to stress that when working with moieties that can sublimate (e.g. camphor, coumarin and menthol), the working temperature must be carefully chosen to avoid undesirable changes in the molar ratio of the constituents. At present, not much information is available regarding HDESs thermal stability and further investigation should be carried out to acquire useful data for better process design.

1.4.4. Solubility in water

The utilization of hydrophilic DES for the recovery of specific moieties from aqueous matrices is actually impossible, due to the potential dissolution of the constituents in water, resulting in DES disruption. Therefore, the evaluation of this property needs accurate investigation to make the extraction system as efficient as possible. HDESs have been studied and formulated to solve the issues arising when using hydrophilic DESs for extractions carried out in an aqueous environment. Actually, in order to retain the DES structure, it is of paramount importance to avoid any contamination or any component dissolution in the aqueous phase. When dealing with ionic HDESs systems, the length of the QAS alkyl chain plays a key role, Influencing the saturated water content. The quaternary ammonium with a long chain shows a higher hydrophobicity than those with a short chain, suggesting that this component, in ionic HDESs, causes the formation of covalent bonds in which are involved water molecules, demonstrating the more useful role that non-ionic HDESs can play in the aqueous system.

1.4.5. Polarity and solvatochromic properties

Polarity represents a useful parameter that gives indications about the interactions that may arise between a given solvent and any possible solute (Reichardt & Welton, 2011). Hence, polarity depends on several different intra- and inter-aggregate/ion pair interactions, namely van der Waals forces, p-interactions and hydrogen bonding. From a practical point of view, an effective way to study intermolecular interaction is represented by the use of solvatochromic probes. In particular, Florindo et al. (2018b) investigated, among others, the polarity of eight different HDESs [tetrabutylammonium chloride/levulinic acid (1:2); tetrabutylammonium chloride/octanoic acid (1:2); tetrabutylammonium chloride/decanoic acid (1:2); tetrabutylammonium chloride/dodecanoic acid (1:2); DL-menthol/levulinic acid (1:1); DL-

menthol/octanoic acid (1:1); DL-menthol/dodecanoic acid (1:2) and /DL-menthol/acetic acid (1:1)], acquiring solvatochromic data and calculating the normalized polarity (ETN) and the Kamlet–Taft parameters values [hydrogen-bond donating ability (α, acidity); hydrogen bond accepting ability (β, basicity); dipolarity/polarizability (π*)]. The obtained results highlighted some interesting points. Regardless of the HBA, the investigated HDESs showed high ETN values indicating how the solvents exhibited hydrophobic and polar characteristics. However, while the values were mainly constant when considering HDESs having the same HBA, significant differences were highlighted when comparing the polarity of solvents obtained with the two HBAs (tetrabutylammonium chloride or DL-menthol). From these results, it can be inferred the key role of HBAs in defining the HDESs polarity. As far as the hydrogen bonding accepting ability (β parameter), despite recording very different values between the two HDESs groups, no significant dissimilarities were observed within each group, pointing out the negligible influence of the various HBDs on the solvent acidity.

The π * parameter gives a measure of the dipolarity and polarizability of a solvent and when compared to hydrophilic DES, the investigated HDESs showed lower π * values, thus being less dipolar and/or polarizable. An appreciable π * difference was also observed between the two HDESs groups, with the DL-menthol-based HDESs showing the lowest π * values. Therefore, the HBA can play an important role in the solvent dipolarity and polarizability, but it must be emphasized how these properties can also be influenced by the HBD moieties nature, being the alkyl chain length inversely related to the π * value.

1.5. Application Applications of HDESs in the food sector

1.5.1. HDESs as extracting media of food contaminants and additives

In recent years, as the living standards have improved, food safety has become a major issue for both consumers and government agencies. Thus, the scientific community is working to develop innovative methods for assessing food contaminants, facing issues related to the use of hazardous and toxic chemicals. Within this frame, the research has been focused also on the use of HDESs, which may represent an interesting green tool for extracting contaminants, harmful moieties, or illegally used additives (Table 1.4).

Table 1.4. Principal applications of HDESs in the food sector

Application	References
Extraction of pesticides and antibiotic residues	Farajzadeh et al., 2017; Florindo et al., 2017 ; Yang et al., 2018; Torbati et al., 2019; Farajzadeh et al., 2019; Deng et al., 2019; Liu et al., 2020; Sereshti et al., 2020; Mogaddam et al., 2020; Lin et al., 2021; Pasupuleti et al., 2022; Cherkashina et al., 2022; Wang et al., 2022; Shirani et al., 2022; Barbayanov et al., 2022; Dal Bosco et al., 2022; Saei et al., 2022.
Extraction of packaging contaminants	Chisvert et al. 2018; Ge et al., 2018; Li et al., 2020a; Naebi et al., 2020; Li et al., 2020b; Ortega-Zamora et al., 2020; Wen et al., 2020; Baute-Pérez et al. 2022.
Extraction of legal or illegal dyes	Zhu et al., 2018; Ghorbani Ravandi & Fat Hi, 2018; Faraji, 2019.; Ahmadi et al., 2019; Liu et al., 2019b; Zhang et al., 2019b; Li et al., 2020c; Sivrikaya Ozak & Yilmaz, 2020.
Extraction of heavy metals	Akramipour et al., 2018; Rad et al., 2019; Sorouraddin et al., 2019; Sorouraddin et al., 2020; Abdi et al., 2020; Elik et al., 2022; Shamsipur et al., 2022; Zhang et al., 2022; Elahi et al., 2022.
Extraction of bioactive compounds	Cao et al., 2017b; Cao et al., 2017a ; Cao et al., 2018; Silva et al., 2019; Dwamena, 2019; Whang et al., 2020; Kanberoglu et al., 2019; Li and Row, 2020; Rodrigues et al., 2020; Triaux et al., 2020; Stupar et al., 2021; Khare et al., 2021; Oliveira et al., 2021; Cañadas et al., 2021; Lazzarini et al., 2022; Liao et al. 2022; Kongpol et al., 2022 .

As far as pesticides, early papers involving the use of HDESs were published in 2017, dealing with the microextraction of nine pesticide residues (bromopropylate, clodinafop-propargyl, diazinon, diniconazole, fenazaquin, haloxyfop-R-methyl, hexaconazole, penconazole and tebuconazole) in vegetable and fruit juice samples (Farajzadeh et al., 2017) and the elimination of four neonicotinoids (acetamiprid, imidacloprid, nitenpyram and thiamethoxam) from diluted aqueous samples (Florindo et al., 2017). In more recent times, Lin et al. (2021) investigated the level of five fungicides (azoxystrobin, cyprodinil, epoxiconazole, fludioxonil, and prochloraz) in fruit juices and tea drinks, using ultrasound-assisted microextraction with HDESs based on L-

menthol and decanoic acid as HBA and HBD, respectively. The authors reported relative recoveries of 71.8-109.4 %; however, these results were obtained working on spiked samples and no direct comparison with other extraction techniques was investigated.

With reference to potential safety issues related to the use of specific food packagings, extraction of phthalic acid esters from water and beverages samples was attempted using HDESs consisting of menthol and acetic acid (Ortega-Zamora et al., 2020) with mean relative recovery values ranging from 71 to 120 % in spiked samples. Recent works also report the utilization of HDESs for detecting food additives, mainly food dyes. In the work of Faraji (2019), two HDESs were prepared by mixing benzyltriethyl ammonium chloride or choline chloride as HBA with thymol as HBD and tested for liquid-liquid microextraction of amaranth, allura red, azorubine, erythrosine and ponceau 4R in beverages, jelly and chocolate dragees. The results showed a better performance than other tested techniques, such as Cloud Point Extraction and Solid Phase Extraction and advantages related to higher simplicity and sustainability of the proposed method.

With regard to the detection of illegal dyes, ten HDESs were used in the vortex-assisted liquid–liquid microextraction of the toxic dye Sudan I (1-phenylazo-2-naphthalenol) in food samples, namely chili oil and sauce, as well as duck egg yolk (Liu et al., 2019b). In the paper, the authors reported that choline chloride/sesamol based HDES allowed better recoveries (93 – 118 %), in comparison with the other tested HDESs and with results reported in the literature obtained using organic solvents.

Regarding other food additives, Zhang et al. (2019a) studied the extraction of nitrites in water and organic fluids as the result of using nitrates as preservatives in meat products. The authors tested HDESs realized with N81Cl) and oleic acid at four different molar ratios. The results showed that when using the N81Cl/oleic acid (1:2) HDES the extraction recoveries ranged from about 90 to 115 %, values not dissimilar to those obtained using the Griess method.

Even for the analysis of heavy metals in foodstuffs, HDESs have shown good performances in the chelating extraction. Sorouraddin et al. (2020) have utilized a ternary HDES system obtained by mixing menthol, sorbitol and mandelic acid (1:2:1) as a simultaneous complexing agent and solvent for the extraction of heavy metal ions (Cd^{2+}, Cu^{2+} and Zn^{2+}) in milk samples spiked with the analytes at three levels (2.5, 7.5, and 15.0 $\mu g\,L^{-1}$ of each cation). The results showed interesting recovery levels, in the range of 88.8 –103.4, 90.1–104.2 and 88.7–101.5 % for Cd2+

Cu^{2+} and Zn^{2+}, respectively. Furthermore, Elik et al. (2022) have utilized HDESs for extraction and preconcentration of Pb^{2+} and Cd^{2+} ions in water (tap, mineral, river and well water) and food samples (sesame, peanut, eggplant, maize, wheat, soybean and cucumber). In their work, the authors initially tested the performance of five different HDESs prepared using DL-menthol, L-menthol, methyl trioctyl ammonium chloride, tetrabutyil ammonium bromide and thymol as HBA, and butyric, decanoic, dodecanoic, oleic and oxalic acids as HBD. The L-menthol/dodecanoic acid HDES resulted to be the most performing one, and its utilization was then optimized with respect to HDES volume, sonication time, extraction temperature, and pH. Water samples were spiked with two-level standard solutions (100 and 200 μg L-1) of Pb2+ and Cd^{2+} and the assessed recoveries ranged from 96.5 to 103.4 % and from 96.4 to 102.5 %, respectively. As far as the food samples, spiked at 50 ppm of each analyte, recovery values for Pb^{2+} and Cd^{2+} were in the range 95.8–102.7 % and 92.8–103.2 %, respectively. While the results seem interesting, the authors did not systematically compare the proposed method and other microextraction techniques, carrying out experiments on the same matrices. An interesting application has been published by Altunay et al. (2019) who investigated the use of HDESs for the extraction of patulin, a mycotoxin, in fruit juices. The study regarded the use of seven DESs obtained combining QASs (choline chloride, tetrabutyl ammonium chloride and tetraoctyl ammonium chloride) as HBA and alcohols (ethylene glycol, glycerol, 1,3-butanediol, 1,4-butanediol, 2,3-butanediol and glycerol) as HBD. After an initial screening, tetrabutyl ammonium chloride/2,3-butanediol (molar ratio 1:2) HDES was selected for testing the extraction performance on apple, orange, peach, apricot, grape, kiwi, cherry and mango juices, which were spiked with patulin at 50 and 200 μg L^{-1} levels. The obtained recoveries ranged from 90.2 to 106.9 %, showing how this extraction procedure could potentially be used in the analysis and quality control of beverages.

1.5.2. HDESs as extracting media of bioactive compounds

Nowadays the recovery of bioactive compounds from natural matrices is usually carried out using organic solvents, but the peculiar characteristics of DESs have sparked interest in investigating their use as an alternative. Most of the studies focus on using hydrophilic DESs, since many biofunctional molecules have hydrophilic characteristics. However, several lipophilic moieties found in natural substrates can also be of significant interest (e.g. carotenoids, tocols). However, as reported in Table 1.4, researchers have mainly dedicated their attention to the use

of HDESs as an extracting media for analytical purposes, while only a limited number of papers have dealt with the use of HDESs as a tool for recovering bioactive compounds. The pertinent results are discussed in the following paragraphs, while a detailed description of the matrices investigated, the extraction conditions, the recoveries, and the equipment used to perform the final quantification are reported in Table 1.5. and Table 1.6.

Table 1.5. Extraction of bioactive compounds from foods, plants and food processing by-products.

	Matrix	Analyte	HDES	Extraction conditions	Method and equipment for quantification (*)	Results (*)	References
Foods	apple, tomato, onion, grape spiked with quercetin: 5 and 12 μg/g in grape; 7.5 and 10 μg/g in onion; 5 and 10 μg/g in tomato; 7.5 and 15 μg/g in apple	quercetin	$N_{4444}Cl$/DecA (1:3)	USAEME with: 300 μL THF 250 μL HDES extraction time 3 min centrifugation at 4500 rpm for 5 min	Spectrophotometric analysis at 370 nm	quercetin recoveries: 91 - 110 %	Kanberoglu et al., 2019
	black pepper (ground pepper and peppercorns samples)	piperine	ChCl/ButA (1:2)	MAE with: sample/solvent ratio 1:200:1 (g/mL); microwave irradiation for 3 min at 10mPa	UHPLC analysis with a C18 column at 340 nm	piperine yields: 35 mg/g in peppercorns 20 mg/g in ground pepper	Dwamena, 2019
	clove, cinnamon, cumin, fennel, nutmeg, thyme	29 terpenes	$N_{4444}Br$/Dodecanol (1:2)	HS-SDME with: extraction temperature 80 °C extraction time 90 min drop volume 1.5 μL sample mass 50 mg	GC-MS analysis	terpenes yields: from 0.47 μg/g (borneol) to 86.40 μg/g (α-farnesene) with more than half of the recoveries under 2 μg/g	Triaux et al., 2020

Table 1.5. Extraction of bioactive compounds from foods, plants and food processing by-products.

	Matrix	Analyte	HDES	Extraction conditions	Method and equipment for quantification (*)	Results (*)	References
	kelp spiked with the selected analytes at concentrations of 5, 100, and 300 μg/mL	D-galactose, L-(-)-fucose, DL-tyrosine DL-valine	two-phase DES system: ChCl/Caffeic acid/Glycerol (1:1:1) as hydrophilic DES and N_{4444}Cl/OctA/ DecA (1:1:1) as hydrophobic DES mixed at a volume ratio of 1:1	AA-DLLME sample/solvent ratio 3:1 extraction temperature 50 °C extraction time 60 min NaCl 10% (w/v) 5 push–pull cycles	HPLC analysis at 330 nm	recoveries: D-galactose 87 - 102% L-(-)-fucose 84 - 103% DL-tyrosine 87 - 104% DL-valine 85 - 103%	Li and Row, 2020

Table 1.5. Extraction of bioactive compounds from foods, plants and food processing by-products.

	Matrix	Analyte	HDES	Extraction conditions	Method and equipment for quantification (*)	Results (*)	References
Plants	*Ginkgo biloba* leaves	flavonoids, PPAs, PAC, TTLs	two-phase DES system: ChCl/LevA (1:2, H_20 40% w/w), ChCl/MalA (1:2, H_2O 55% w/w) as hydrophilic DESs and $N_{81}Cl$/Capryl alcohol/OctA (1:2:3) as hydrophobic DES mixed at a volume ratio of 35:5:40	stirring method: stirring rate 150 rpm sample/solvent ratio 1:20 (g/mL) extraction temperature 65 °C extraction time 42 min	flavonoids: spectrophotometric method at 510 nm; PPAs: HPLC analysis with a C18 column at 215 and 256 nm PAC: spectrophotometric method at 644 nm; TTLs: spectrophotometric method at 517 nm	first extraction rates: - PAC 86% - Flavonoids 78% - TTLs 93% - PPAs 95% (calculated carrying out a second extraction step on the pellet under the same optimal conditions)	Cao et al., 2018

Table 1.5. Extraction of bioactive compounds from foods, plants and food processing by-products.

	Matrix	Analyte	HDES	Extraction conditions	Method and equipment for quantification (*)	Results (*)	References
	Curcuma longa L. rhizomes, leaves and flowers	antioxidant compounds	Men/LacA (1:2); Men/AcA (1:1)	UAE with: sample/solvent ratio 1:20 (g/mL) extraction temperature 45 °C extraction time 2-3 h according to the substrate	DPPH, FRAP and TFC analyses	DPPH, FRAP and TFC results for the 3 substrates: 4 to 62 mg/g of Trolox, 30 to 90 mg/g of ferric sulfate and 3 to 11 mg/g of quercetin in Men/LacA extract; 2 to 42 mg/g of Trolox, 60 to 83 mg/g of ferric sulfate and 5 to 17 mg/g of quercetin in Men/AcA extract	Oliveira et al., 2021
	Curcuma Longa L. rhizomes	curcumin, bisdeme-thoxycurcumin, demethoxycurcumin ar-turmerone	OctA/L-Men (1:3.6) + Tween 80:PG(1:1) + water at the volume ratio 30:60:10	UAE with: sample/solvent ratio 10:1 (mg/mL) extraction time 90 min	HPLC analysis at 425 and 240 nm	extraction yields: - curcumin 2 % - bisdemethoxcurcumin 6% - demethoxycurcumin 13% - ar-turmerone 3% (w/w, dry basis)	Kongpol et al., 2022
Food processing by-products	tomato processing by-product (pomace)	lycopene	DL-Men/LacA (8:1)	UAE with: sample/solvent ratio 1:120 (g/mL) extraction temperature 70 °C extraction time 10 min	Spectrophotometric analysis at 477 nm	lycopene yield: - 1447 μg/g of dry tomato pomace	Silva et al., 2019

Table 1.5. Extraction of bioactive compounds from foods, plants and food processing by-products.

	Matrix	Analyte	HDES	Extraction conditions	Method and equipment for quantification (*)	Results (*)	References
	brown crab and shrimp shells	astaxanthin	Men/MyrA (8:1)	SLE with: sample/solvent 1:0,25 (g/g) extraction temperature 60 °C extraction time 2 h	HPLC analysis at 478 nm for acetone and 484 nm for Men/MyrA extracts	astaxanthin yields: - similar [9.3 μg/ g (d.w.)] in brown crab shells - increased up to 657-fold in the remaining biomasses with reference to Soxhlet extraction for 6 h with acetone	Rodrigues et al., 2020
	winery wastewater (synthetic samples obtained diluting 5 times with distilled water a red wine aliquot)	phenolic compounds	$N_{8881}Cl$/DL-Men (1:2); $N_{8881}Cl$/OctA (1:1)	LLE with: sample/solvent ratio 1:1 extraction time 15 min at 500 rpm centrifugation time 15 min at 3500 rpm	TPC: Folin Ciocalteu method and spectrophoto-metric quantification at 765 nm; antioxidant activity: DPPH free radical method HPLC analysis at 257 nm for 4-hydroxybenzoic acid, 271 nm for gallic acid and syringic acid, 309 nm for p-coumaric acid and 323 nm for caffeic acid and ferulic acid	extraction recoveries: - $N_{8881}Cl$/DL-Men (1:2) 83.64% - $N_{8881}Cl$/OctA (1:1) 84.10% (calculated by subtracting the remaining TFC in the diluted sample after the extraction to the TFC value in the initial sample without treatment)	Cañadas et al., 2021

Table 1.5. Extraction of bioactive compounds from foods, plants and food processing by-products.

	Matrix	Analyte	HDES	Extraction conditions	Method and equipment for quantification (*)	Results (*)	References
	tomato processing by-product (pomace)	lycopene and β-carotene	DL-Men/LacA (8:1)	UAE with: solvent/sample ratio 120:1 (mL/g) extraction temperature 63 °C extraction time 20 min	Spectrophoto-metric analysis at 477 nm for lycopene and 461 nm for β-carotene	extraction recoveries: lycopene 102 and 109% β-carotene 61 and 74% with reference to n-hexane:acetone and acetate:ethyl lactate extractions	Lazzarini et al., 2022

AA-DLLME = air assisted dispersive liquid–liquid microextraction; ChCl = choline chloride; DecA = decanoic acid; DL-Men = DL-menthol; DMAC = 4-(dimethylamino) cinnamaldehyde; DoDecA = dodecanoic acid; GC-MS = gas chromatography mass spectrometry; HPLC = high performance liquid chromatography; HS-SDME = headspace single-drop microextraction; LacA = lactic acid; LevA = levulinic acid; LLE = liquid–liquid extraction; MAE = microwave-assisted extraction; MalA = malonic acid; Men = menthol; MyrA = myristic acid; N4444Br = tetrabutylammonium bromide; N4444Cl = tetrabutylammonium cloride;N81Cl = methyl trioctyl ammonium chloride;N8881Cl = trimethyloctylammonium chloride; NaCl = sodium chloride; OctA = octanoic acid; PAC = procyanidine; PPAs = polyprenyl acetates; SLE = solid−liquid extractions; TFC= total flavonoid content; TPC = total phenolic content; THF = tetrahydrofuran; TTLs = terpene trilactones (TTLs); UAE = ultrasound assisted extraction; UHPLC = ultra high performance liquid chromatography; USAEME = ultrasound-assisted emulsification microextraction.

(*) not all the published papers reported in detail the data.

Table 1.6. Extraction and recovery of bioactive compounds from foods and plant matrices.

Matrix	Analyte	HDES	Extraction conditions	Method and equipment for quantification and recovery (*)		Results (*)		References
				Quantification	Recovery	Extraction step	Recovery step	
Ginkgo biloba leaves	polyprenyl acetates	N_{81}Cl/Caprylalcohol/OctA (1:2:3)	stirring method: - stirring rate 150 rpm - sample/solvent ratio 1:11 (g/mL) - extraction temperature 61 °C - extraction time 35 min	HPLC analysis with a C18 column, detection at 210 nm	- adsorption using 6 macroporous resins: HPD-17, D101, DM130, HPD-450, ADS-17, AB-8 - desorption using ethyl acetate	extraction yield: - from 99 to 117% with reference to n-hexane, ethyl acetate and petroleum ether extractions	polyprenyl acetates recoveries: 77% with resin DM130 74% with resin AB-8	Cao et al., 2017b (**)

Table 1.6. Extraction and recovery of bioactive compounds from foods and plant matrices.

Matrix	Analyte	HDES	Extraction conditions	Method and equipment for quantification and recovery (*)		Results (*)		References
				Quantification	Recovery	Extraction step	Recovery step	
Ginkgo biloba leaves	flavonoid glycosides, terpene lactones and biflavonoids	two-phase DES system: Men/AcA (1:1) as hydrophobic DES and water as hydrophilic phase, with a final volume ratio of 1:1	UAE: - sample/solvent ratio 15:1 (mg/mL) - power 200 W - extraction time 10 min	UHPLC-QQQ-MS/MS LC-MS/MS analysis	two-phase extracts were obtained at 10000 rpm: - from the aqueous phase flavonoid, glycosides and terpene lactones were recovered in AcA by drying the extracts - using vacuum distillation, the organic solvent was separated from the biflavonoids and reutilized for 3 other extraction cycles	extraction recoveries: - from 52 to 270% with reference to methanol extraction	main recoveries: ginkgetin 268% isoginkgetin 235% sciadopytisin 100% no significant differences were found among 3 extraction cycles for the targeted molecules, except for rutin, kaempferol-3-O-rutinoside and bilobetin	Wang et al., 2020

Table 1.6. Extraction and recovery of bioactive compounds from foods and plant matrices.

Matrix	Analyte	HDES	Extraction conditions	Method and equipment for quantification and recovery (*)		Results (*)		References
				Quantification	Recovery	Extraction step	Recovery step	
Artemisia annua leaves	artemisinin	$N_{81}Cl$/1-butanol (1:4)	UAE: - solvent/solid ratio 17.5:1 - particle size 80 mesh - power 180 W - temperature 45 °C - extraction time 70 min	HPLC analysis detection at 292 and 260 nm	- adsorption using 6 macroporous resins: HPD-17, D101, DM130, HPD-450, ADS-17, AB-8) - desorption using methanol, 90% methanol, ethanol, 90% ethanol, ethyl acetate and petroleum ether - the HDES was used for up to 3 extraction cycles	artemisinin yield: - 129% with reference to petroleum ether extraction	recoveries using resin AB-8: - 82% with ethanol desorption - 88% with ethyl acetate desorption	Cao et al., 2017a (**)
mushroom	ergosterol	L-Men/PyrA (1:2)	stirring for 120 min UAE: - sample/solvent ratio 1:20 (g/mL) - sonication time 45 min	HPLC analysis detection at 265 nm	ergosterol rich phase, after decantation for 24 h and cetrifugation for 10 min, was purified by adding water to the system and washing with hexane	ergosterol extraction yield 7 mg/g (d.w.)	ergosterol purity 90%	Khare et al., 2021

Table 1.6. Extraction and recovery of bioactive compounds from foods and plant matrices.

Matrix	Analyte	HDES	Extraction conditions	Method and equipment for quantification and recovery (*)		Results (*)		References
				Quantification	Recovery	Extraction step	Recovery step	
pumpkin	β-carotene	Caprylic acid/Capric acid (3:1)	UAE: - sample/solvent ratio 1:7 (g/mL) - power 53 W - temperature 60 °C - extraction time 10 min	HPLC analysis detection at 450 nm	β-carotene recovery from the solution was obtained by precipitation, switching the solvent polarity (addition of water and NH_4OH)	β-carotene extraction: 135 μg/mL	86 % recovery of β-carotene in the precipitate	Stupar et al., 2021
Taraxacum mongolicum and *Lonicerae japonicae Flos*	caftaric acid, chlorogenic acid, caffeic acid, cichoric acid, 3,5-diO-caffeoylquinic acid in *Taraxacum mongolicum* chlorogenic acid in *Lonicerae japonicae Flos*	Men/p-chlorophenol (1:3)	HDES-assisted water extraction: - sample + HDES and water (20:80) (solid–liquid ratio 1:50) - vortexing time 10 min	HPLC analysis with a C18 column; detection at 335 nm	two-phases were obtained by centrifugation: - aqueous phase, containing the phenolic acids; - HDES phase (tested for other 3 extraction cycles)	- phenolic acids yield: 14 mg/g working on *Taraxacum mongolicum* samples - chlorogenic acid yield: 110 % with reference to methanol extraction, working on *Lonicerae Japonicae Flos* samples	extraction efficiency remained over 65% in the 3 cycles, with losses of HDES during each cycle	Liao et al. 2022

Table 1.6. Extraction and recovery of bioactive compounds from foods and plant matrices.

Matrix	Analyte	HDES	Extraction conditions	Method and equipment for quantification and recovery (*)		Results (*)		References
				Quantification	Recovery	Extraction step	Recovery step	

AcA= acetic acid; HPLC = high performance liquid chromatography; L-Men = L-menthol; Men = menthol; $N_{81}Cl$ = methyl trioctyl ammonium chloride; OctA = octanoic acid; PyrA = pyruvic acid; UAE = ultrasound assisted extraction; UHPLC = ultra-high performance liquid chromatography triple quadrupole mass spectrometry.

(*) not all the published papers reported in detail the data.

(**) the recovery method requires the use of organic solvents.

1.5.2.1. Extraction from foods matrices

In 2019 Kanberoglu et al. published the first paper on the extraction of bioactive moieties from foods matrices investigating the use of two HDESs for extracting quercetin from apple, tomato, onion, and grape matrices (Table 1.5.). HDESs were prepared using tetrabuthylammonium chloride/decanoic acid and tetrabuthylammonium bromide/decanoic acid and the influence of type, composition, and volume of the selected HDESs was evaluated so to identify the optimal working conditions. Grape, onion, tomato, and apple samples were spiked with different levels of quercetin, namely 5 and 12 μg/g in grape, 7.5 and 10 μg/g in onion, 5 and 10 μg/g in tomato, and 7.5 and 15 μg/g in apple. Ultrasound-assisted emulsification microextraction was performed using tetrabutylammonium chloride/decanoic acid (1:3) as HDES, obtaining recoveries ranging from 91 to 110%. Although the results showed the potential of the tested method, no comparison with other classical extraction techniques was reported. In the same year, Dwamena (2019) proposed the design of choline-chloride and fatty acids HDESs, studying in depth their physicochemical properties and assessing the efficiency of the piperine extraction from black pepper (ground pepper and peppercorns samples). The prepared and then selected HDESs were choline chloride/butyric acid 1:2, choline chloride/valeric acid 1:2, choline chloride/caprylic acid 1:2. The experiments were carried out using microwave-assisted extraction (MAE) or subcritical (SCE) extraction techniques, even if regarding the latter, the author did not report the critical temperature and pressure values of the investigated HDESs. Comparison among the different HDESs systems, revealed that choline chloride/butyric acid allowed obtaining the most interesting results, being able to extract 70 % of the piperine found in black pepper. Conversely, the experimental data showed how SCE performed slightly better than MAE when working with black pepper, while no substantial differences could be highlighted for peppercorns.

Using as substrate Kelp, a staple food in the Asiatic diet, Li & Row (2019) studied the use of a hydrophilic–hydrophobic DES system for the concurrent extraction of hydrophilic monosaccharides and hydrophobic amino acids (D-(þ)-galactose, L-(-)-fucose, DL-tyrosine DL-valine). Three hydrophilic DESs [choline chloride/caffeic acid (1:2), choline chloride/glycerol (1:2), choline chloride/caffeic acid/glycerol (1:1:1)] and three HDESs [tetrabutylammonium chloride/octanoic acid (1:1); tetrabutylammonium chloride/decanoic acid (1:1); tetrabutylammonium chloride/decanoic acid/octanoic acid (1:1:1)] were utilized for the

preparation of the solvent and their efficiency was tested at eleven different hydrophilic:hydrophobic DES ratios, ranging from 10:0 to 0:10. The maximum yield of the target molecules was obtained with the combination of choline chloride/caffeic acid/glycerol (1:1:1) and tetrabutylammonium chloride/octanoic acid/decanoic acid (1:1:1) at the volume ratio 1:1, and extracting the sample for 60 min at 50 °C. An ionic additive, namely NaCl, was also added because of its ability in enhancing analytes transfer into the hydroalcoholic phase.

Working with spices, Triaux et al. (2020) tested the use of HDESs as a tool for the extraction of terpenes from clove, cinnamon, cumin, fennel, nutmeg and thyme. Ten HDESs were prepared using tetrabutylammonium bromide, methyltrioctylammonium chloride and choline chloride as HBAs, and four alcohols (butanol, octanol, decanol, dodecanol), two acids (hexanoic and lactic acids) and urea as HBDs. Among the tested solvents, the HDES based on N4444Br and dodecanol (1:2) showed the highest performances, and the extraction process was then optimized for analytical purposes to be utilized for headspace single-drop microextraction (HS-SDME) of volatile compounds. Extraction temperature and time, as well as mass sample and drop volume, were the parameters taken into account. While the Authors reported that the DES-HS-SDME method allowed obtaining extract concentrated in a wide range of terpenes and terpenoids, no comparison was made with other extraction solvents/techniques so that the real advantage of using an extraction step based on the use of HDES could not be clarified.

1.5.2.2. Extraction from plants matrices

In order to satisfy the increasing demand for dietary food supplements, the industry is looking for new and efficient extraction techniques for recovering bioactive compounds from natural substrates, mainly represented by plants. Over the past four years, a few papers have been published that examine the efficiency of HDESs in the extraction from Ginkgo biloba and Curcuma longa L. (Table 1.5). In 2018, Cao et al. developed a two-phase DES system to obtain the simultaneous extraction from Ginkgo biloba leaves of different bioactive compounds, characterized by heterogeneous polarities [flavonoids, polyprenyl acetates (PPAs), procyanidine (PAC) and terpene trilactones (TTLs)]. One hydrophobic and two hydrophilic DESs composed the two-phase DES system, and during the extraction, the apolar PPAs moved into the hydrophobic phase, while TTLs, flavonoids and PAC were partitioned into the hydrophilic one. The obtained results showed an excellent capacity of the newly prepared DESs system in recovering the extraction of the entire set of targeted compounds, with recoveries ranging from

78 to 95 %. In another work, Oliveira and co-workers (2021) investigated the extraction of antioxidant compounds from rhizomes, leaves and flowers of Curcuma longa L.. The ultrasound-assisted extraction was performed using three HDESs (menthol/lactic acid 1:2, menthol/lauric acid 2:1, menthol/acetic acid 1:1) and two DESs (choline chloride/lactic acid 1:1, choline chloride/acetic acid 1:1). For comparison, the recovery of the bioactive molecules was carried out also with ethanol, an organic solvent frequently utilized for the extraction of bioactive compounds. The different solvents were initially screened at 35 °C taking into account flavonoid yield and antioxidant properties, the latter being measured using DPPH and FRAP tests. Two HDESs, menthol/lactic acid and menthol/acetic acid, were then selected because of their performances, and the extraction process was further optimized for temperature (45 °C) and extraction time (2-3 h according to the substrate). The biological properties of the extracts were then tested, both in vitro and in vivo, assessing their iron chelation capacity, antibacterial activity and cholinergic activity, while the potential cytotoxicity and genotoxicity were tested on root meristem cells of Allium cepa L., so to highlight any possible threat related to the use of the HDESs extracts in foods. Interestingly, the chelating capacity of the pure menthol/lactic acid and menthol/acetic acid HDESs resulted to be not statistically different and higher than that of the obtained extracts. In particular, while the menthol/lactic acid extracts showed a chelating capacity ranging from 58 to 90 % of that exhibited by the pure solvent, when using the menthol/acetic acid HDES the chelating capacity of the extracts fell dramatically, being reduced to a mere 4 % in case of rhizome extracts, or to a maximum of 60 % in case of the flower extracts. However, it should be pointed out that while exhibiting relatively higher chelating capacity, the flower extracts showed also the lowest antioxidant activity and concentration in flavonoids. As far as antibacterial and cholinergic activities, the extracts obtained from flowers and leaves showed interesting properties in inhibiting food spoilage bacteria. Among the tested microorganisms, three gram-positive (Listeria monocytogenes, Clostridium perfringens, Staphylococcus aureus) and two gram-negative (Escherichia coli and Salmonella sp.) bacteria resulted significantly affected, thus showing the possibility of interesting applications of HDESs extracts in food preservation, as well as in realizing edible coatings. Furthermore, while the menthol/lactic acid extracts exhibited high acetyl and butyryl cholinesterase inhibition properties, ranging from 82 to 99 % in comparison to the control, the extracts obtained by using the menthol/acetic acid DES showed much lower inhibitory

activities, ranging from 6 to 38 %. Of the utmost interest are the obtained data regarding the cytotoxicity and genotoxicity of the HDESs extracts. Working on Allium cepa root cells, a widely accepted substrate for toxicogenetic tests, none of the extracts exhibited potential toxicity issues, showing how their use as such might be considered safe, and highlighting the great potential of extracts obtained by using HDESs for different applications in the food, cosmetic and pharmaceutical industry. Lately, Kongpol and co-workers (2022) utilized the same matrix as the substrate for the extraction of curcuminoids (bisdemethoxcurcumin, demethoxycurcumin and curcumin) and ar-turmerone using a HDES-based microemulsion. The authors tested several HDESs, combining L-menthol as HBD with different fatty acids as HBAs (octanoic acid/L-menthol, decanoic acid/L-menthol and dodecanoic acid/ L-menthol) at several molar ratios (2:1, 1:1, 1:2). The octanoic acid/L-menthol (1:2) HDES showed the highest extraction efficiency, with recoveries ranging from 24 to 86 % for the four analytes, and was then selected for the subsequent optimization step. The optimized operating conditions were identified as: solvent to sample ratio 1:10 (mL/mg), HBA:HBD molar ratio 1:3.6, and extraction time 90 min.

Considering the interesting yields obtained, the authors investigated also the possibility of using the HDES as a carrier of curcuminoids and turmeric oils in foods. This idea was based on the hypothesis that a HDES-based microemulsion could facilitate the dispersion of curcuminoids and ar-turmerone in an aqueous solvent and therefore in foods with high water content. Working on this assumption, five different HDES/surfactant mixtures based on the use of Tween, propylene glycol (PG) and Labrasol (a nonionic oil-in-water surfactant used as a solubilizer in topical formulations) were tested for their abilities to extract the compounds of interest. While all systems were found to be suitable extractants, the highest recoveries were obtained using the microemulsion system HDES/Tween 80:PG (1:1)/water at the ratio 30:60:10. Interestingly, the addition of water increased the recovered amounts of curcuminoids and ar-turmerone even if these moieties are water insoluble and further investigation is needed to clarify this behavior.

1.5.2.3. Extraction from food processing by-products

The agro-food industry produces large amounts of wastes and by-products with a remarkable environmental impact. The implementation of circular economy models may reduce this negative characteristic, transforming wastes and by-products into new sources of viable

compounds, which may find use in several industrial applications. Within this frame, the use of HDESs has triggered the attention of the academic community and a few papers have been published dealing with the production of extracts of interest for the food, cosmetic and pharmaceutical industry (Table 1.5). In 2019, Silva et al. proposed the extraction of lycopene from tomato pomace, an industrial by-product made of seeds and skins, using food grade HDESs. After some screening tests, the HDES based on DL-menthol/lactic acid was chosen because of the higher lycopene recoveries in comparison with other HDESs based on choline-chloride and levulinic acid. The extraction process was optimized with reference to temperature, HBA:HBD molar ratio, solvent:sample ratio and time. The maximum lycopene yield (1447 μg/g dry tomato pomace) was obtained working at 70 °C, HBA: HBD molar ratio = 8:1, solvent:sample = 120 mL/g and processing time = 10 min. This result is of interest, being the assessed yield about 8 % higher than that obtained working with ethyl acetate. However, it should be stressed that while the extraction could be carried out in a very short time, the solvent:sample ratio may represent an important drawback when scaling up the process, due to the large amounts of HDES needed per unit of the substrate. Based on the results obtained by the group of Silva, in a very recent paper, Lazzarini et al. (2022) utilized the same HDES, DL-menthol/lactic acid (8:1), for the extraction of lycopene and β-carotene from the same substrate, comparing the extraction yields with those obtained using n-hexane/acetone (1:1) and ethyl acetate/ethyl lactate (70:30), the latter being considered an eco-friendly solvent. The efficiency of the various solvents was also investigated with reference to three different drying procedures utilized to stabilize the raw material before the extraction step (heat drying, freeze-drying and non-thermal air-drying). The reported data indicate how the best results were obtained on air-dried samples, and in comparison with n-hexane/acetone, the HDES allowed lycopene and β-carotene recoveries of about 102 and 61 %, respectively. These percentages increased up to 109 and 74 % in comparison with the yields obtained using ethyl acetate/ethyl lactate. Despite these good results, the authors indicated how the excessive viscosity of the tested HDES might represent a drawback, affecting its extraction capability. Always referring to the extraction of carotenoids, Rodrigues et al. (2020) studied the potential use of terpene-based natural HDESs for astaxanthin extraction from brown crab shell residues. The authors investigated the use of several compounds for preparing the solvents, and eucalyptol and camphor were chosen as HBAs, while perillyl alcohol, DL-menthol and myristic acid were utilized either as HBAs or as

HBDs, due to their dual behaviour. The study involved also the evaluation of other important extraction parameters, such as time and temperature, carrying out the tests within the range 30-60 °C and 2-24 h, respectively, while keeping constant the sample/solvent ratio (1:4). Among the various solvents tested, the highest recoveries were obtained using the HDES consisting of menthol/myristic acid (8:1) and extracting the substrate at 60 °C for 2 h. The assessed yields were not significantly different from those obtained with acetone extraction under reflux for 6 h. The authors also investigated the recovery of astaxanthin from shrimp shells, mussels and microalgae (*Haematococcus pluvialis*), always in comparison to the results obtained by using acetone. While in the case of shrimp shells no significant differences could be highlighted, when working with mussels or microalgae significant recovery increases were observed, being the obtained astaxanthin yields 3 to 657 fold higher, respectively. Recently, Cañadas et al. (2021) described the use of HDESs to recover the phenolic antioxidant fraction from winery wastewater. In particular, by using ammonium salts, DL-menthol and fatty acids, fourteen hydrophobic eutectic mixtures were prepared and tested for liquid–liquid extraction of the moieties of interest. In comparison with ethyl acetate, the new solvents allowed to attain higher yields. Specifically, when working with the organic solvent only about 15 % of the available phenolic compounds was extracted, while when using trimethyloctylammonium chloride/DL-menthol (1:2) or trimethyloctylammonium chloride/octanoic acid (1:1) the recoveries were much higher, about 84 % in both cases. However, it should be emphasized how the authors carried out the research using synthetic winery wastewater, obtained diluting a red wine sample with distilled water (1:5 v/v) to simulate the wastewater concentration resulting from a winemaking process. This may represent a major drawback of the study, being the obtained solution not representative of real-life wastewater.

1.5.2.4. Extraction and recovery from foods and plants matrices

When setting up an extraction process, the recovery of the extracted moieties from the extraction solvent is a fundamental and challenging step, because of the interest in obtaining pure extracts and recycling the extraction media. However, when dealing with HDESs only a very limited number of papers facing this problem can be found in the literature.

In particular, Cao et al. (2017b) described the use of a methyltrioctylammonium chloride/capryl alcohol/octanoic acid (1:2:3) HDES for the extraction of polyprenyl acetates from Ginkgo Biloba leaves. The recovery of the pure compounds was investigated testing the use of six

different macroporous resins (HPD-17, D101, DM130, HPD-450, ADS-17 and AB-8) for the adsorption/desorption of the moieties. The resins and the laden HDESs were shaken at 150 rpm and 25 °C for 12 h, and the polyprenyl acetates concentration in the supernatant was then quantified to assess the adsorption yields. Afterward, the polyprenyl acetates-laden resins were filtered out and the compounds were desorbed using ethyl acetate (Table 1.6.). The experimental data indicated that the resins DM130 and AB-8 allowed for obtaining the highest recoveries of the target compounds, with yields of 76.52 and 74.08 %, respectively.

In another study, the authors applied the same experimental approach for recovering artemisinin from extracts obtained treating Artemisia annua leaves with a HDES realized with methyltrioctylammonium chloride/1-butanol (1:4) (Cao et al., 2017a). In this case, the best results were obtained with the resins ADS17 and AB-8, while the desorption step was carried out testing different eluents (methanol, 90% methanol, ethanol, 90% ethanol, ethyl acetate and petroleum ether). The process was then optimized with regard to the loading of the resin (resin/HDES solution 0.45 g/mL), reaching an artemisinin adsorption yield of 85.65 %. The most performing elution solvents resulted to be ethanol and ethyl acetate, with assessed desorption yields of 96.32 and 103.11 %, respectively and total artemisinin recoveries of 82 and 88 %. As the last step, the authors investigated the HDES recyclability, using the same solvent in three subsequent extractions. The results did not highlight any significant difference between the first and the second cycle, while when used for the third time the HDES showed a noticeable decrease in its extracting power, up to 54 % of the initial one. The tested procedure and the resultant recoveries might rise some interest, particularly when distillation or crystallization cannot be utilized to recover the extracted molecules, due to the low vapor pressure of the HDES components. However, it should be pointed out that the proposed method requires the use of organic solvents, in contrast with the aim of environmental sustainability for which the HDES was used in the extraction step. For the extraction of phytochemicals from Ginkgo Biloba, Wang et al. (2020) have proposed the use of a two-phase DES system made of menthol/acetic acid (1:1) and water at the volume ratio 1:1, in comparison with the use of water and two conventional organic solvents, namely methanol and ethanol. Eleven compounds of interest were identified and quantified, and according to their nature, they were partitioned into either the water or the DES phase, which were subsequently separated by centrifugation. From the water phase the extracted flavonoids, glycosides and terpene lactones were recovered

removing the solvent under vacuum, while the bioflavonoids were recuperated from the menthol phase by vacuum distillation. With reference to the use of methanol, the reported extraction efficiency of the two-phase DES system ranged from 52 to 270 % according to the molecule of interest (Table 1.6.). The authors also investigated the possibility of recycling the extraction solvents and the recovered menthol oils were then reutilized for three cycles. The experimental data showed that the extraction efficiency remained high over the whole set of extractions, even if a general trend to a slight decrease could be highlighted. In particular, the assessed decrease for ginkgetin, isoginkgetin and sciadopytisin, the most abundant compounds found in the extracts, ranged from about 8 to 13 %. A higher decrease, about 40 %, could be detected for rutin and bilobetin, however it should be pointed out that their concentration in the extracts was around 2 orders of magnitude lower than that of ginkgetin, isoginkgetin and sciadopytisin. In 2021, Khare et al. investigated the recovery of ergosterol from mushroom by testing thirty-nine HDESs prepared using various combinations of menthol, thymol or tetrabutylammonium hydrogen bromide as HBAs and various long chain acids as HBDs. The best results were obtained using a HDES made of L-menthol/pyruvic acid (1:2), with an extraction yield of about 89 % (Table 1.6.). In the paper, the authors claimed to be able to recover the ergosterol molecules by simply letting the mixture stand still for 24 h and then applying a centrifugation step (1000 rpm, 10 min). The supernatant fraction, represented by the HDES containing around 10 % ergosterol, was then reused for up to six subsequent extraction cycles, with a solvent loss of about 10 % per cycle and an extraction efficiency of about 28 % at the end of the sixth cycle. The ergosterol-rich phase was instead added with water and then washed with hexane. After the removal of the organic phase, the ergosterol was obtained at a purity level of over 90%. While the proposed procedure can rise some interest, is it not clear how the ergosterol separation might occur by using a mere centrifugation step. In the paper, it is not reported that the extraction step should be carried out at high temperature so to hypothesize a natural precipitation of the extracted ergosterol due to a loss of solubility at room temperature. Furthermore, despite obtaining ergosterol at a very high purity level, the use of hexane represents a major drawback when aiming to reduce/eliminate organic solvents.

Always in 2021, Stupar et al. investigated the ultrasound-assisted extraction of β-carotene from pumpkin, screening ten HDESs and selecting the one made of caprylic acid/capric acid (3:1) (Table 1.6.). In the study, the authors also tackled the problem of recovering the carotenoid from

the solvent, suggesting a strategy based on switching the solvent polarity to obtain the β-carotene precipitation. The procedure involved adding the obtained HDES extract with water, hence causing the formation of two phases due to the different polarities of the various moieties. Afterward, by adding a weak base, namely NH4OH, the pH of the system was modified, causing a modification of the HDES polarity and the mutual dissolution of the two previously obtained phases. This polarity modification allowed the recovery of the extracted β-carotene by spontaneous precipitation, induced by the much lower solubility of the compound in a polar system. From a practical point of view, the proposed approach for lipophilic compounds recovery after extraction looks promising, and the authors suggested that the resultant switched solvent could be either used for extracting hydrophilic biomolecules or switched back to the initial hydrophobic characteristics by simply adding CO_2 to the solvent system.

Recently, a new approach to using HDESs has been reported by Liao et al. (2022). The authors investigated the use of a hydrophobic deep eutectic solvent-assisted water extraction method, for recovering five phenolic acids (caftaric acid, chlorogenic acid, caffeic acid, cichoric acid, 3,5-diO-caffeoylquinic acid) from Taraxacum mongolicum, so to obtain an extract claimed to be suitable for the pharmaceutical industry. The extraction procedure was realized by adding both the water and the HDES to the powdered substrate, mixing and then centrifuging, so to obtain a two-phase liquid–liquid system, represented by the hydrophobic phase and the aqueous phase, rich in phenolic acids. In the study, thirty-four different HDESs were tested for their efficiency in enhancing the phenolic acids, and the camphor/p-chlorophenol (1:3) HDES resulted to be the most performant (Table 1.6.), with recoveries about 15-25 % higher than those obtained using methanol or ethanol, and almost threefold higher with respect to using just water as a solvent. However, the mechanisms underlying the observed extraction efficiency enhancement have not yet been clearly elucidated. The authors suggested that an intermolecular hydrogen bond might have been generated between the HDES chloride anion and the phenolic acids, hence causing better extraction from the substrate. Thereafter, the higher affinity of the acidic moieties with water would have caused their partitioning into the polar phase. As an additional point, the density of the investigated p-chlorophenol-based HDES resulted to be higher than that of water, and this was reported as being a key point for better dispersion of the substrate between the aqueous phase and the HDES phase, allowing better extraction efficiencies. The authors investigated also the aspects related to the solvent recyclability. The HDES was then

recovered at the end of the extraction procedure and reutilized twice, for a total of three extraction cycles. As far as the extraction efficiency, no significant difference could be highlighted throughout the cycles for the phenolic acids considered. Conversely, the HDES amounts recuperated showed that at the end of each cycle a significant amount of HDES was not recovered, with a total final loss of about 35 % at the end of the third cycle. Despite the authors reported the possible use as such of the phenols-enriched aqueous phase for pharmaceutical purposes, the presence in the extracts of p-chlorophenol based HDES residues may represent a health issue, being the exposure to this chemical associated with problems involving the nervous system. Thus, further studies are required to evaluate the potential hazards to human health.

1.6. Limitations and future challenges

The green chemistry and its principles were introduced in the late 1990s, aiming to reduce the use and generation of harmful substances, mostly through promoting innovative research in the field of sustainable technology. Due to their sustainability, easy preparation, versatility, and relatively low price, the HDESs have enormous potential as green solvents. However, one of the main drawbacks is represented by a limited knowledge of the mechanisms underlying the physicochemical properties shown by these solvents, in particular considering HDESs prepared with natural moieties. Actually, the majority of the data available and herein revised are referred to HDES made with synthetic materials. The lack of data regarding the solid-liquid equilibria makes it difficult to fully understand the behavior of these solvents, especially with regard to their solidification, which can occur even for small variations of the components' molar ratio (van Osch et al., 2020). Furthermore, their effective hydrophobicity may also be considered a controversial aspect, taking into account that some HDESs reported in the literature are made up of notoriously non-hydrophobic components (acetic acid, lactic acid and short-chain QASs), which thus may interact with the water eventually present in the treated substrates. Another problem that should be stressed is the lack of standardized methods for the separation phase, that allow simultaneously the solvent recycling and the target biomolecules recovery, two aspects of utmost importance for the scale-up in the food sector. A separation method currently experienced is the so-called back extraction, which would require the use of an additional solvent to separate DESs from the extracted biomolecules. To date, this system does not appear to be the most suitable method because of its inner irreconcilability with the aims of green chemistry

and the inevitable additional costs, which can represent a problem in industrial applications. Among the reported works dealing with the setup of a separation method, the one proposed by Stupar et al. (2021) is very promising. The method is based on the use of a switchable-hydrophilicity solvent (SHS), a field initially investigated by several authors for non-DES systems (Jessop et al., 2010; Boyd et al., 2012; Samorì et al., 2014). The authors suggested as the DES polarity could be reversed from hydrophilic to hydrophobic by adding CO_2 to the system, similarly to what was proposed by Cicci et al. (2018), when processing microalgae with N,N-dimethyl-cyclohexylamine as a switchable solvent. However, this potential procedure needs to be further deeply investigated to evaluate the recovery efficiency and the operating conditions needed when working with different starting materials for HDESs preparation and extracting biomolecules of various nature. As proposed by Cao et al. (2019b), another method that could be of interest is represented by the use of resins; however, the additional costs and recovery rates need to be evaluated. Therefore, the scientific community's efforts are pivotal for developing innovative methods to improve the operating conditions of this fundamental phase of recycling/purification. Focusing on the possible use of the extracted bioactive compounds for food applications, an additional solution could be represented by the use of natural HDESs, which being obtained combining non-harmful moieties, may find a direct use for the formulation of enriched and fortified foods. Nowadays, ready-to-use food grade extracts are highly required, and their production should not imply additional purification steps, being the latter costly and time-consuming (Chemat et al., 2012). Nevertheless, the direct application of NADESs extracts in food formulations is still little or not explored. In addition, the use of this specific subclass of HDESs, for their inner nature, should not have an impact on the related environmental and sustainability issues, even if the deepening of toxicity and biodegradability aspects should be investigated for each type of natural HDES.

Taking together, natural HDESs may represent a very interesting group of solvents for the extraction of natural compounds for food applications, starting from raw materials or food wastes and by-products. Due to their peculiar characteristics, HDESs may be utilized for recovering apolar moieties, such as carotenoids, lipophilic polyphenols, vitamins, essential fatty acids, or even polar ones when combined with other suitable non-toxic solvents as water. A comparison between the extraction efficiency obtained using natural hydrophobic and hydrophilic DES on the same matrices and targeted molecules could be useful to evaluate the

practical advantage in the use of the former. Therefore, further studies are needed to exploit the potentialities of natural HDESs, focusing, among other issues, also on the recyclability problems, in order to reach their practical utilization at industrial level.

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2. Green Extraction of Carotenoids from Vegetable by-products Using Natural Hydrophobic Deep Eutectic Solvents

The content of this chapter was already published in:

26th Workshop on the *Developments in the Italian PhD Research on Food Science Technology and Biotechnology*, University of Turin, Asti (2022). Sportiello, L. Optimization of the extraction techniques using Deep Eutectic Solvents for the recovery of biomolecules from food industry by-products. Poster.

2.1. Introduction

The agri-food industry generates a large amount of by-products, representing an important issue of global interest. This is principally due to vast amounts of organic moieties such as proteins, lipids, and carbohydrates, which can negatively affect the environment and human health (Ezejiofor and Uchechi, 2014). The by-product composition may also include valuable bioactive compounds, as reported by several studies (Ben-Othman et al., 2020; Chaouch and Benvenuti, 2020; Skendi et al., 2020), which can provide health benefits, such as antihypertensive, anticancer, anti-inflammatory, hypoglycemic, antimicrobial, antiviral, antitumor, antithrombotic, hypocholesterolemic, etc.

Recently, the importance of these substances and their beneficial effects on human health has gained particular attention due to the spread of diet-related diseases and the increasing consumers' interest in a healthy lifestyle. In this context, the transition from a linear to a circular economy in the agri-food sector can be promoted by implementing models aimed at the extraction and recovery of valuable molecules from the available by-products before their utilization as an energy source or mere disposal (Donner et al., 2020). In addition, due to increasing environmental concerns, there has recently been an increasing interest in developing green extraction technologies to be used in the food industry. According to the principles of Green Chemistry, reducing or eliminating toxic organic solvents represents one of the significant issues (Sportiello et al., 2023). Within this frame, the use of NaHDESs as green substitutes for conventional organic solvents has gained increasing interest in the last eight years since their introduction in 2015 (van Osch et al., 2015). This field has significantly evolved in the last few years, showing a great diversity of starting materials used in NaHDESs preparation, including terpenes and medium-long alkyl chain carboxylic acids (Florindo et al., 2017).

In this work, eleven NaHDESs already described in the literature (Mako's et al., 2018; Křížek et al., 2018; Hummer et al., 2018; Silva et al., 2019; van Osch et al., 2019) and composed by DL-menthol, thymol, camphor, and lactic and decanoic acid were subjected to a physicochemical characterization, acquiring

data not yet present in literature. After that, their extraction efficiency was evaluated for the recovery of carotenoids from four processing by-products, namely the peels of fresh carrots, yellow and red peppers, and pumpkins. Once the best performers for each matrix were selected, the ultrasound-assisted extractions were optimized using the Box-Benhken Design (BBD) combined with the Response Surface Modelling (RSM).

2.2. Materials and methods

2.2.1. Standards, reagents and solvents

DL-menthol (≥ 98.0 %), camphor (> 96 %), thymol (≥ 98.5 %), decanoic acid (≥ 98.0 %), and lactic acid (>90 %) were used for the NaHDESs preparation. Furthermore, acetone (≥99.8 %), methanol (≥99.9 %), β-carotene (96.4 %), and lutein (95.7 %) were used for the HPLC analysis. All the chemicals were purchased from Merck KGaA (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

2.2.2. Matrices preparation

The by-products, namely the peels derived from the processing of fresh carrots, yellow and red peppers, and pumpkins, were kindly supplied by Ortonuovo Srl (Arbizzano-Santa Maria, VR). The collected samples were cleaned with tap and ionized water and then dried with absorbent paper. After removal of the seeds and comminution, the samples were freeze-dried using a LIO-5P DGT lyophilizer (Vetrotecnica, PD, Italy) and afterward grounded using a Polymix PX-MFC 90D mill (Vetrotecnica, PD, Italy). The resulting powder was vacuum-sealed and stored at -20 °C.

2.2.3. NaHDESs preparation

Eleven NaHDESs were prepared according to the method proposed by Dai et al. (2013), with slight modifications. The HBA and the HBD were combined in a specific molar ratio and mixed at 750 rpm under mild heating at 60 °C until a clear transparent liquid was formed. In the case of thymol-based NaHDESs, pale yellow transparent liquids were obtained. Afterward, the resulting solvents were gradually cooled to room temperature, and their stability was monitored during storage.

2.2.4. NaHDESs characterization

The prepared NaHDESs were physicochemically characterized in terms of density, dynamic viscosity, and rheological behavior as well as by Fourier Transform Infrared-Attenuated Total Reflection (FTIR-ATR) Spectroscopy.

2.2.4.1. Density

NaHDESs density was measured utilizing a pycnometer (Merck KGaA, Darmstadt, Germany). The measurements were carried out in the temperature range 20-80 °C. The density/temperature dependence was described using the following equation (Equation 2.1):

$$\rho = a + bT \qquad (Eq. 2.1)$$

Where ϱ is the density in grams per cubic centimeter, T is the temperature in °C, and a and b are the fitting parameters. The experimental density results as a function of temperature and the adjustable parameters (a and b) were determined from fitting the experimental density data to Eq. 2.1 and are reported in Table S2.1 and S2.2, respectively, in the Supplementary Section.

2.2.4.2. Viscosity and rheological behavior

The rheological characteristics of the prepared NaHDESs were evaluated using a DSR 500 CP4000 rheometer (Lamy Rheology, Champagne-au-Mont-d'Or, France). In all cases, viscosity measurements were carried out using the measuring system MK-SV418, applying different shear-rates, ranging from 50 to 300 s-1. The viscosity values were fitted to the Power Law model as a function of shear-rate as described by Equation 2.2 to calculate the flow behavior (n) and flow consistency (K) indices (Macosko, 1994).

$$\eta(\dot{\gamma}) = K \cdot \dot{\gamma}^{n-1} \qquad (Eq. 2.2)$$

Where η is the viscosity (mPa·s), *K* is the flow consistency index (mPa·s) representing the viscosity at the shear-rate $\dot{\gamma} = 1\ s^{-1}$, and *n* is the power law index (adimensional) defining the steepness of the shear thinning decay for $n < 1$ (Eberhard et al. 2019). Furthermore, measurements were also conducted in the temperature range of 20-60 °C, and the obtained values were fitted as a function of temperature using the Vogel-Fulcher-Tammann (VTF) model (Eq. 2.3):

$$\ln \eta = A_\eta + \frac{B_\eta}{(T - C_\eta)} \qquad (Eq. 2.3)$$

where η is the viscosity in mPa·s, T is the temperature in K, and Aη, Bη, and Cη are adjustable parameters. The experimental results of viscosity as a function of temperature and the parameters are presented in Table S2.3 and Table S2.4 in the Supplementary Section.

2.2.4.3. Fourier Transform Infrared-Attenuated Total Reflection (FTIR-ATR) spectroscopy analysis

The individual starting material, as well as all the investigated NaHDESs, were analyzed using a Thermo Scientific FTIR spectrometer (Class 1 Laser Product Nicolet 6100, San Jose, CA). The equipment included ATR accessories with a diamond crystal of 42° for solids and a zinc selenide crystal of 45° for liquids. The spectra were acquired using the OMNIC 7.3 software (Thermo Electron Corporation). Before the acquisition, a background spectrum was recorded and used as a reference. The spectra of each sample were then recorded at room temperature between 4000 and 400 cm^{-1} by placing them in the corresponding ATR crystal. The final spectrum was obtained by averaging 32 individual scans, each with a resolution of 4 cm^{-1}.

2.2.5. NaHDESs screening for the extraction of carotenoids

For the extraction tests, an aliquot (0.1 g) of lyophilized sample was added to 5 mL of each NaHDES (sample:solvent 1:50 w/v), vortexed at 25 °C for 60 seconds and then kept under continuous mixing for 30 minutes using a disc rotator (UniLOPMIX2, LLG-Labware, Meckenheim, Germany). Afterwards, the mixture was sonicated for 60 minutes at 45 kHz (2200 MH S3, SOLTEC, Milan, Italy) before being centrifuged at 3900 RCF for 10 minutes. All manipulations were carried out shading the samples to minimize carotenoid photodecomposition throughout the analytical procedure. The extraction under the same experimental conditions using acetone as the extraction solvent was also carried out to compare the extraction efficiency of the investigated NaHDESs with that of a conventional organic solvent. Furthermore, exhaustive extractions were conducted to assess the extraction efficiency of each solvent in comparison with the maximum theoretical yields. All extractions were performed in triplicate, and the results were expressed as the mean value ± standard deviation.

2.2.6. Extraction efficiency determination

The extraction efficiency of the carotenoids derived from the four selected matrices, namely the peels of carrot, yellow and red peppers, and pumpkins, was monitored using UV-Vis spectrophotometry and HPLC-DAD analysis to quantify the yields of β-carotene and lutein.

2.2.6.1. UV-Vis spectrophotometry

The Beer-Lambert law was used to assess the total carotenoid content of the NaHDESs extracts at 450 nm as described by Ordonez-Santos et al. (2019) using a V-750 UV-Visible Spectrophotometer (Jasco, Oregon, USA). The samples were diluted with acetone (1:5 v/v), and the investigated solvents were used

as a blank for each extract. Carotenoids were quantified using the extinction coefficient for β-carotene in acetone (2500) and expressed as mg of β-carotene/mL of extract.

2.2.6.2. HPLC-DAD analysis

β-carotene for pumpkin peel extracts, lutein for yellow pepper peel extracts, and both β-carotene and lutein for carrot and red pepper peel extracts were determined using a LC-4000 HPLC system equipped with an MD-4010 PDA Detector (Jasco, Oregon, USA) and a C_{30} column (4.6 × 250 mm, 5 μm, YMC Inc., Wilmington, NC). Peak separation was obtained working in solvent gradient mode, using solvent A (H_2O/MeOH 20/80 by volume) and solvent B (acetone/MeOH 1:1 by volume) at the flow rate of 1 mL/min with the following gradient profile: 25% B 0–4 min; 100% B 4–10 min; 100% B 10–25 min; 25% B 25–36 min, according to the procedure previously described by Seregelj et al., 2019, slightly modified. Analytical samples were diluted in acetone (1:2 by volume) and filtered through a 0.45 μm PTFE filter (Frisenette, Knebel, Denmark). β-carotene and lutein were identified by comparing the retention times and spectral characteristics (absorption maxima) with those of external standards. Additionally, pigment quantification (mg/mL of extract) was carried out by using the external standard technique and specific calibration curves ($y = 39931x + 60766$, $R^2 = 0.996$, 0.625–20 μg of β-carotene /10 μL, with seven points; $y = 58415x – 5872.2$, $R^2 = 0.995$, 0.156–5 μg of lutein/10 μL, with six points).

2.2.7. Experimental design for the extraction processes optimization

After the preliminary screening analysis, three NaHDESs were identified as the best performers for the extraction from the selected four matrices. In order to optimize the operating conditions for maximizing the extraction efficiency for each matrix, four three-factor, three-level BBD combined with RSM were implemented. In this study, the effect of HBA:HBD molar ratio (X_1), the solvent to sample ratio (X_2) and the extraction time (X_3) were selected as independent variables or factors, investigated at three different levels and coded as -1, 0 and +1. Furthermore, according to the data reported by Purohit and Gogate (2015) and Stupar et al. (2021), the extraction temperature was fixed at 50 °C. The experimental range and levels of the independent variables of each BBD are listed in Table 2.1. Variables X_2 and X_3 were studied at the same three levels for all the extraction processes, while for variable X_1, different levels were identified according to the different NaHDESs selected during the screening step.

Table 2.1. Experimental ranges with the coded and natural values of the independent variables for carotenoid extraction from carrot and yellow pepper peels (HDES 6), red pepper peels (HDES 9), and pumpkin peels (HDES 2).

Independent variables	Levels		
	-1	0	-1
(X_1) HBA:HBD molar ratio			
HDES 6	0.25	3	5.75
HDES 9	0.5	1.5	2.5
HDES 2	0.25	4	7.75
(X_2) Solvent:sample ratio (v/w)	10	30	50
(X_3) Extraction time (min)	30	60	90

The total carotenoids, spectrophotometrically calculated, and the yields of β-carotene and lutein, obtained by HPLC analysis, were selected as dependent response variables to be optimized in the four extraction processes. A second-order model was adopted to explain the relationship between the dependent responses and the factors using the following quadratic equation (Eq. 2.4):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} x_i x_j + \varepsilon \qquad Eq.(2.4)$$

where Y is the response variable, k is the number of the patterns, i and j are the index numbers for pattern, β_0 is the 'intercept term', x_i, x_j,..., x_k are the coded independent variables, β_i and β_{ii} are respectively the linear and the quadratic coefficients, β_{ij} is the interaction effect, while ε is the random error explaining differences between predicted and measured values. The β-carotene and lutein extraction efficiency from the vegetable by-product samples was assessed by HPLC-DAD analysis, as previously reported. The software Design Expert (Version 8.0.7.1, Stat-Ease Inc., MN, USA) was used to set seventeen batch experiments with five central points for each BBD. The extraction tests were conducted in triplicate to ensure model strength except for the central points.

2.2.8. Statistical analysis

Statistical analysis of the data obtained during the screening step was performed using the software XLSTAT Premium (Version 2020.3.1, Addinsoft, Paris, France), applying one-way ANOVA. Significant differences between means were computed by Tukey's HSD (Honestly Significantly Different) test at 95% confidence interval. In addition, fitting of the Equation 2.3 was obtained using the software OriginPro (Version 2023, OriginLab Corporation, Northampton, Ma, USA), while the FTIR-ATR

spectra were acquired using the software OMNIC 7.3 (Thermo Electron Corporation, Madison WI, USA). For the process optimization, the dependent variables were analyzed using the BBD. The final models included terms with a significance level below 0.05 ($p < 0.05$), as well as those necessary to maintain the hierarchical structure of the model. Multiple linear regression analysis was conducted to create the best-fitted model. The adequacy of the model was evaluated based on the model p-value, lack of fit, coefficient of determination (R^2), and the adjusted coefficient of determination (R^2_{adj}). According to the desirability function, the optimal extraction conditions were calculated to maximize the extraction efficiency of all target responses simultaneously and apply constraints for the variables X_2 (solvent:sample ratio) and X_3 (extraction time) to minimize the process costs. The adequacy of the model was verified by carrying out extractions at the predicted optimal conditions in three replicates. The experimental design, the optimization step, and the construction of three-dimensional response surface plots were obtained using the software Design Expert software (Version 8.0.7.1, Stat-Ease Inc., MN, USA).

2.3. Results and discussion

2.3.1. NaHDESs preparation and characterization

In the present study, eleven different NaHDESs composed of natural and food-grade substances, i.e., monoterpenes (DL-menthol, thymol, camphor) and carboxylic acids (lactic acid and decanoic acid) were prepared at specific molar ratios, reported in Table 2.2, together with the physicochemical characterization and the extraction efficiency of the formulated solvent. NaHDESs composed exclusively of a combination of DL-menthol and thymol were also prepared since these terpenes can act simultaneously as HBAs and HBDs due to the presence of the OH group (van Osch et al., 2019; Cao et al., 2021). The prepared solvents were then characterized for their physicochemical properties, highly variable according to the solvent composition, due to the significant impact of these characteristics on the extraction efficiency. In particular, density is one of the most important physical properties when considering the performance of a solvent because it can be used in thermodynamic models and process simulations that are required to study mass transfer, heat transfer, etc. (Lemaoui et al., 2020). All the density values obtained are in agreement with those reported in the literature for NaHDESs composed of monoterpenes and carboxylic acids (Martins et al., 2018; Makoś et al., 2018; Lalikoglu et al., 2022). For the investigated NaHDESs, the density was assessed as lower than that of water in all cases, except for HDES 2, and in particular ranged from 0.858 to 1.031 g/cm^3 at 25 °C, substantiating the difference with the hydrophilic DESs density, that is generally higher than that of water (~1.15 g/cm^3) (van Osch et al., 2019). The recorded highest density value of the HDES 2 is due to the presence of lactic acid used in

solution at 90 % and its amount in the solvent (DL-menthol/lactic acid 1:2). The lowest density (0.858 g/cm^3) was observed for DL-menthol and decanoic acid NaHDES (HDES 4) at a molar ratio of 1:1.

As far as viscosity, the obtained values ranged from 18.862 to 134.688 mPa•s at 25 °C. The data showed how all the NaHDESs, except HDES 3, fulfill 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). Generally speaking, hydrophobic DESs are reported to be less viscous than hydrophilic ones, a feature that enhances mass transfer and their applications as extraction solvents (Cao et al., 2021). Furthermore, according to the literature (Tang et al., 2021; Sportiello et al., 2023), the viscosity values of the investigated solvents were found to be higher for menthol-based NaHDESs than thymol-based NaHDESs.

In addition, as reported in the previous section, the NaHDESs viscosity was assessed in the shear-rate range 50 – 300 s^{-1}. This investigation was carried out to acquire information on the rheological flow behavior of these solvents and their efficiency as extraction solvents. Actually, in the literature, there is no agreement concerning the shear flow behavior of Natural hydrophilic and hydrophobic DESs, since they are reported as non-Newtonian fluids, with a shear-thickening or shear-thinning behavior depending on the range of shear-rate applied (Altamash et al. 2017; 2018; Mišan et al., 2019; Kyriakoudi et al., 2022). For this study, the obtained data showed that the prepared NaHDESs exhibit a shear-thinning behavior in the imposed shear-rate range, with a recorded viscosity decrease when higher values of shear-rate were applied. A fluid's flow behavior, calculated according to Eq. (3), is generally described as shear thinning for $n < 1$, shear-thickening if $n > 1$, or Newtonian flow for $n = 1$. The experimental "n" values calculated using Equation (2.3) (see Section 2.2.4.2) are reported in Table 2.2.

Table 2.2. HBAs/HBDs, molar ratios, physicochemical characterization of the prepared NaHDESs, and carotenoid content of the obtained extracts (mg/mL) as determined by UV-Vis spectrophotometry after extraction with different NaHDESs during the screening step.

NaHDESs	HBA/HBD	Molar Ratio	Density* (g/cm³ at 25 °C)	Viscosity* (mPa·s at 25 °C)	Flow Behavior Index (n) *	Flow Consistency Index (K) * (mPa·s)	Total Carotenoids (450 nm) **, ***,#			
							Carrot Peels	Yellow Pepper peels	Red Pepper Peels	Pumpkin Peels
							mg β-Carotene/mL			
HDES 1	DL-menthol/lactic acid	1:1	0.981	56.712	0.702	49.621	1.041 ± 0.007 ^{e}	0.262 ± 0.001 ^{f}	0.853 ± 0.020 ^{j}	0.710 ± 0.022 ^{d}
HDES 2		1:2	1.031	54.821	0.731	42.192	0.623 ± 0.010 ^{g}	0.339 ± 0.001 ^{de}	1.495 ± 0.044 ^{gh}	1.165 ± 0.003 ^{b}
HDES 3		8:1	0.898	134.688	n.a.	n.a.	0.840 ± 0.001 ^{f}	0.313 ± 0.010 ^{ef}	1.367 ± 0.036 ^{h}	0.830 ± 0.006 ^{c}
HDES 4	DL-menthol/decanoic acid	1:1	0.894	35.241	0.455	35.214	1.071 ± 0.006 ^{de}	0.395 ± 0.002 ^{c}	1.753 ± 0.076 ^{ef}	0.256 ± 0.004 ^{g}
HDES 5		6.5:3.5	0.921	31.782	0.683	57.173	1.213 ± 0.021 ^{c}	0.420 ± 0.017 ^{c}	1.081 ± 0.019 ^{i}	0.211 ± 0.012 ^{hi}
HDES 6	thymol/DL-menthol	1:1	0.935	37.863	0.671	33.201	1.338 ± 0.007 ^{b}	0.473 ± 0.014 ^{ab}	2.312 ± 0.024 ^{ab}	0.193 ± 0.005 ^{i}
HDES 7		1:2	0.924	54.685	0.753	27.329	1.281 ± 0.007 ^{b}	0.427 ± 0.026 ^{bc}	1.953 ± 0.018 ^{cd}	0.113 ± 0.005 ^{j}
HDES 8	thymol/decanoic acid	1:1	n.a.	n.a.	n.a.	n.a.	1.026 ± 0.146 ^{e}	0.317 ± 0.014 ^{e}	2.104 ± 0.164 ^{c}	0.241 ± 0.004 ^{gh}
HDES 9		3:2	0.919	18.862	0.705	52.625	1.216 ± 0.155 ^{c}	0.389 ± 0.024 ^{cd}	2.365 ± 0.010 ^{a}	0.352 ± 0.003 ^{f}
HDES 10	camphor/decanoic acid	1:2	0.931	25.586	0.661	25.894	1.202 ± 0.028 ^{c}	0.414 ± 0.015 ^{c}	1.574 ± 0.016 ^{fg}	0.132 ± 0.004 ^{j}
HDES 11		1:1	n.a.	n.a.	n.a.	n.a.	1.155 ± 0.002 ^{d}	0.478 ± 0.015 ^{ab}	1.830 ± 0.023 ^{de}	0.478 ± 0.019 ^{e}
Acetone							1.612 ± 0.023 ^{a}	0.479 ± 0.013 ^{a}	2.114 ± 0.071 ^{bc}	1.240 ± 0.027 ^{a}

* Results are expressed as the mean value of two independent measurements. ** Data with different superscripts in the same column are significantly different according to Tuckey's test at $p < 0.05$. *** Results are expressed as the mean value of three independent experiments ± standard deviation. # Expressed as β-Carotene.
n.a. = not assessable.

Furthermore, as described in the previous sections (2.2.4.1 and 2.2.4.2), density and viscosity data were also acquired within the temperature range of 20−80 °C for the former and 20-60 °C for the latter. The data reported in Table S2.1, S2.2, and S2.3 and depicted in Figure 2.1 and 2.2. As can be observed, due to their instability, the investigated properties of HDES 8 and HDES 11 were not assessable in many cases.

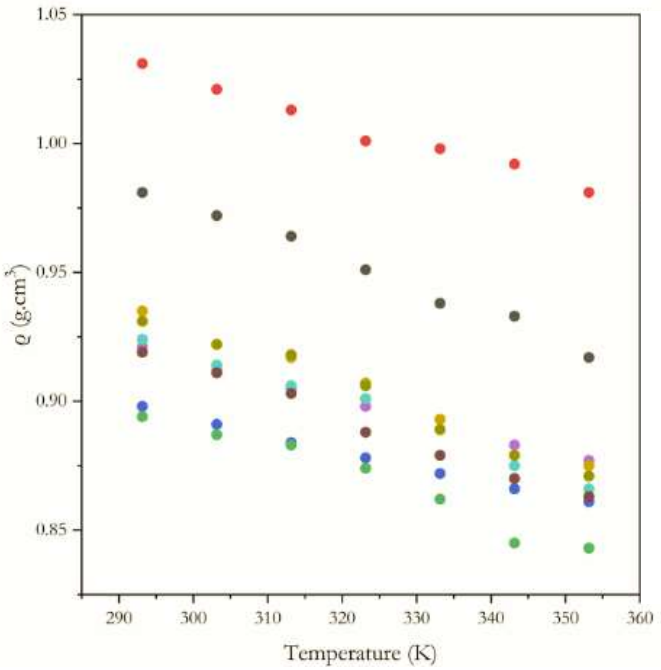


Figure 2.1. Experimental densities of the tested NaHDESs as a function of temperature (black= HDES 1; red= HDES 2; blue= HDES 3; green= HDES 4; violet= HDES 5; yellow= HDES 6; aquamarine= HDES 7; brown= HDES 9; dark green=HDES 10).

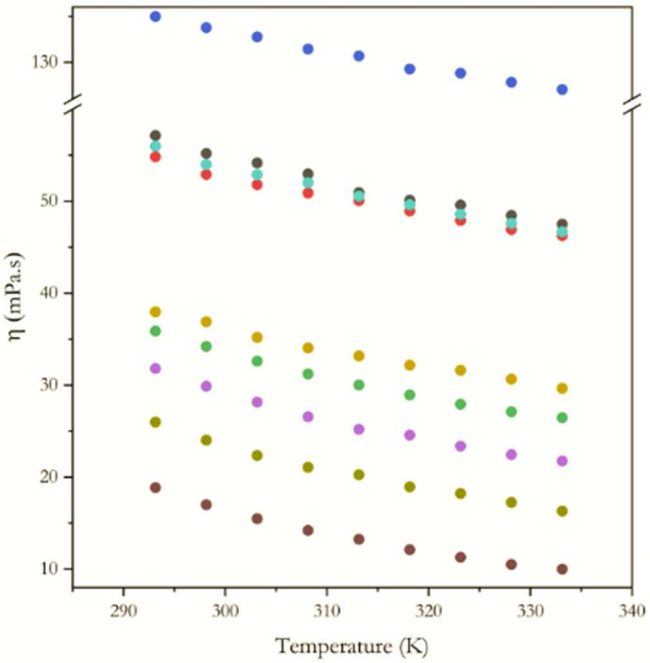
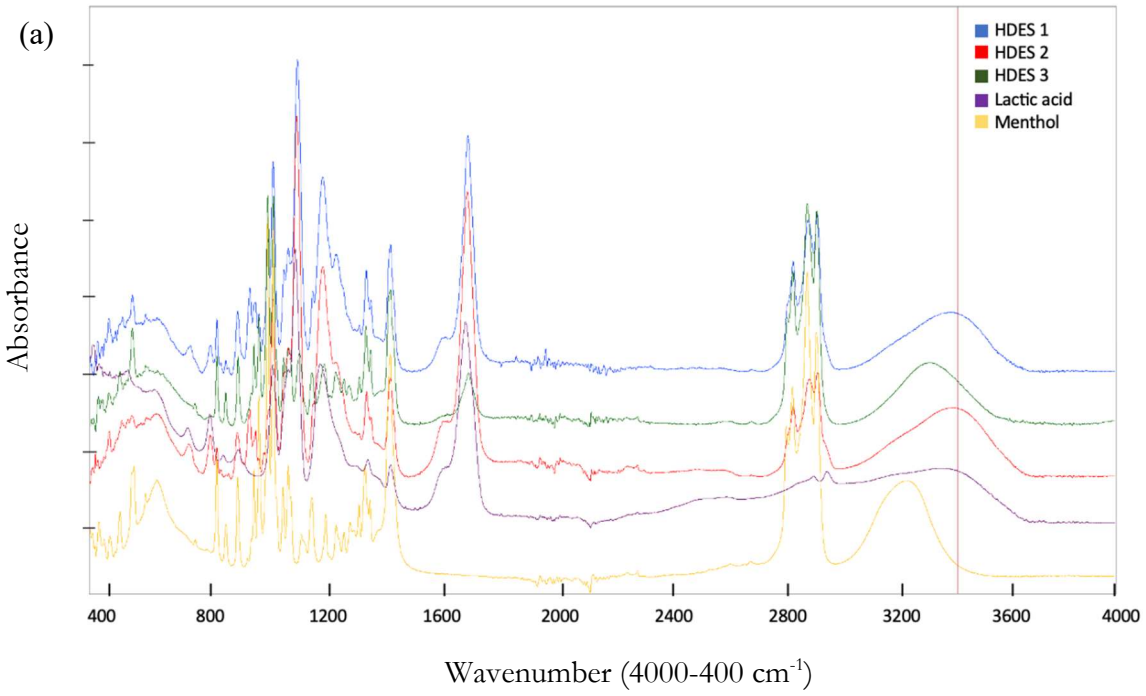
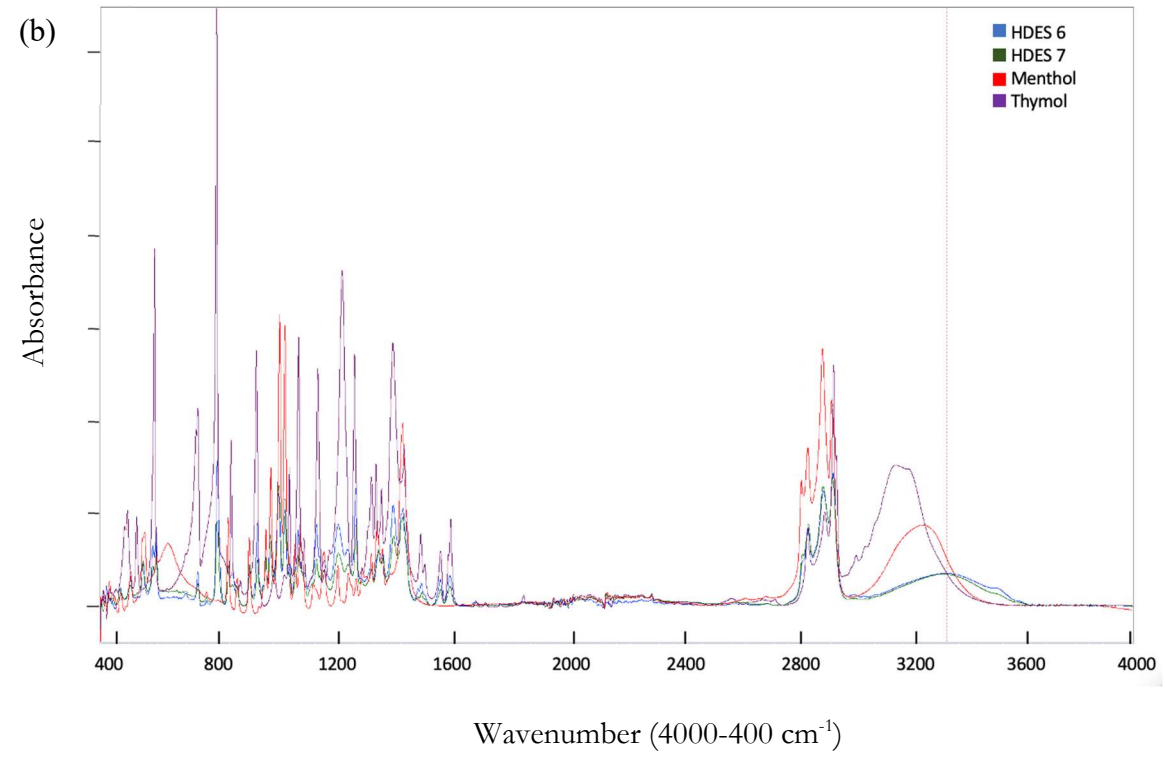


Figure 2.2. Experimental viscosities of the tested NaHDESs as a function of temperature (black= HDES 1; red= HDES 2; blue= HDES 3; green= HDES 4; violet= HDES 5; yellow= HDES 6; aquamarine= HDES 7; brown= HDES 9; dark green=HDES 10).

Lastly, in this study, FTIR-ATR spectroscopy was utilized to investigate the molecular interactions among the individual components of the prepared NaHDESs. This technique was found to be particularly useful in confirming the formation of hydrogen bonds between HBA and HBD (Shafie et al., 2019).





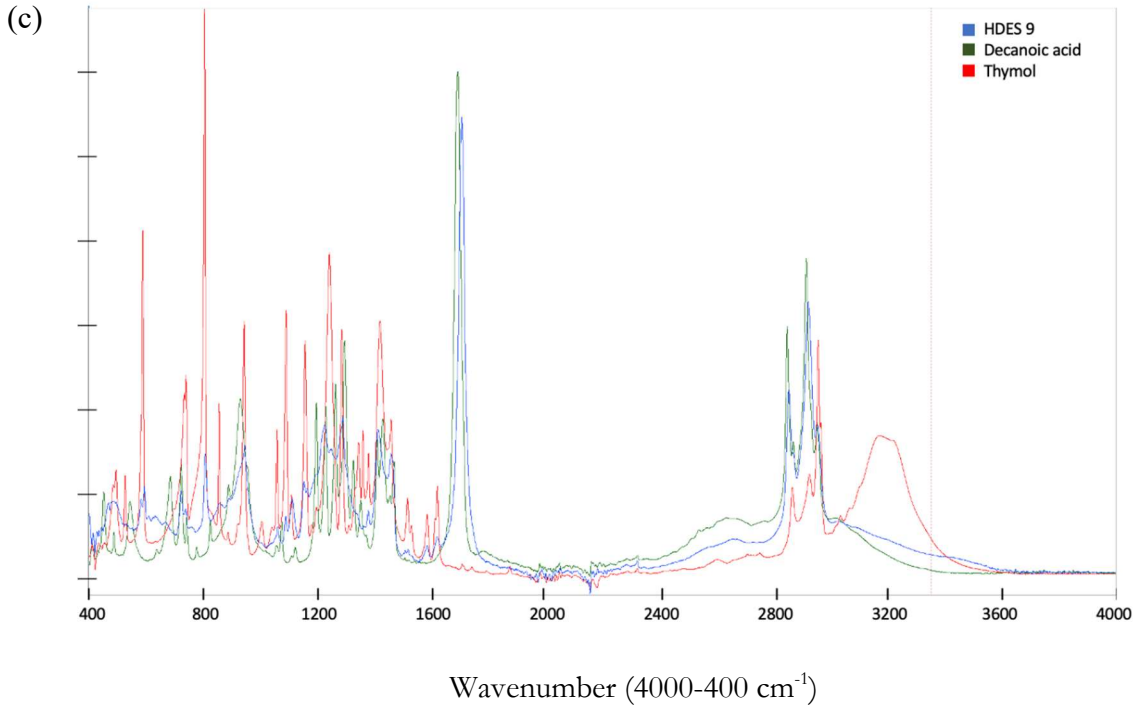


Figure 2.3. FTIR-ATR spectra of NaHDESs and their individual components. (a) HDES 1, HDES 2, HDES 3, DL-menthol, and lactic acid; (b) HDES 6, HDES 7, thymol, and DL-menthol; (c) HDES 9, DL-menthol, and thymol.

The obtained FTIR-ATR spectra provided noteworthy information, especially observing those of HDES 1, HDES 2, and HDES 3 (Fig. 2.3 a), HDES 6 and HDES 7 (Fig. 2.3 b), and HDES 9 (Fig. 2.3 c). In all cases, the mixtures showed absorption profiles similar to those of the individual components, except for the O−H stretching vibration bands. Interestingly, these bands were found to be shifted to lower frequencies (higher wavenumbers) when compared to the pure components of the mixture, confirming the formation of hydrogen bonding through the hydroxyl groups (Rodrigues et al., 2020; Kyriakoudi et al., 2022). In particular, when considering HDES 1, 2, and 3 (Fig. 2.3 a), in the HDES 2 spectra the OH band (3300-3500 cm^{-1}) is the one most shifted to high energy. Between HDES 6 and HDES 7 (Fig. 2.3 b), the same observation can be reported for HDES 6 and, lastly, even HDES 9 (Fig. 2.3 c) displayed a more intense shoulder at 3450 cm^{-1} with reference to the pure components. In this case, the shift towards higher energies of the mode to 1700 cm^{-1} (C=O bond) is also observable, further indicating the hydrogen-bond network establishment between the individual components in the course of the successful NaHDESs formation.

2.3.2. Selection of the best performing NaHDESs

The prepared NaHDESs were tested for their efficiency in extracting carotenoids from carrot peels, yellow and red pepper, and pumpkin. Extraction under the same experimental conditions using acetone as the extraction solvent was also carried out as a reference. Furthermore, exhaustive extractions were conducted to compare each solvent's extraction efficiency to the maximum extraction yield obtainable from each matrix. The extraction efficiency of the investigated NaHDESs is shown in Table 2.3. Although all the eleven solvents were tested in the screening step, a first selection was made observing their stability during storage after their preparation. HDES 3, 8, and 11 showed thermal instability with the tendency to separate when cooled below 25 °C, giving rise to two layers and requiring subsequent heating for their use as extraction media. Therefore, they were excluded from the subsequent optimization step. The extraction recoveries, expressed as mg β-Carotene/mL of extract, were statistically evaluated using ANOVA to assess differences and to identify the best-performing NaHDESs for each matrix. In general, compared to the extractions carried out using acetone, some of the green solvents tested allowed recoveries higher than 80 % for all four matrices, with values over 100 % when working with the red pepper peels. In the case of the carrot by-products, HDES 6 and 7 showed to be the most efficient extracting media, with values of total carotenoids significantly higher than all the other NaHDESs. Furthermore, they allowed to recover 12 % of the theoretically available carotenoids and around 83 % of the amount obtained working with acetone under the same conditions. It should be pointed out that these two solvents were prepared using the same HBA and HBD (thymol/DL-menthol) at different molar ratios, thus giving reason to select this combination of starting materials for the extraction optimization. The HDES 6 was also chosen for the extraction from yellow pepper peels, allowing the highest recovery, corresponding to about 99 %, compared to the extraction with acetone. Actually, no significant differences were found between the extraction efficiency of this solvent and that of the HDES 11. Still, despite the excellent performance shown, the latter was discarded for instability issues. In the case of red pepper peels, the choice was oriented toward HDES 9, even if the assessed recovery was not significantly different at $p<0.05$ from that of HDES 6 (112 and 109 % of the total carotenoids obtained with acetone, respectively). This selection was made to deepen the extractive capability of a DES made up of thymol and decanoic acid, which are less expensive than the components used for preparing HDES 6 and characterized by a less intense menthol aroma. Furthermore, HDES 2, prepared by combining DL-menthol and lactic acid at 1:2 molar ratio, was selected as the best-performing solvent for the extraction from pumpkin peels, allowing recoveries of about 94 % in comparison with

the acetone extraction, and 25 % in comparison with the carotenoids amount obtained in the exhaustive extraction.

2.3.3. Optimization of the extraction processes

The operating conditions applied in the extraction technique are crucial parameters for obtaining processes that can be implemented at the industrial level, as they strongly influence their costs and efficiency. Furthermore, NaHDES composition plays a key role in establishing interactions with the targeted molecules, influencing its extraction capability. In order to maximize the extraction of carotenoids, avoiding waste of resources, in the present study the effect of HBA:HBD molar ratio, solvent:sample ratio, and extraction time on the content of total carotenoids and the yields of β-carotene and lutein was investigated using BBDs coupled with RSM and the results for each matrix are reported in Tables 2.3, 2.4, and 2.5. On average, the data obtained were higher than those observed in the screening step, except for the yellow pepper peels matrix. Although the matrices used in the two experimental phases belonged to the same sampling, a possible fluctuation in the carotenoid content can be considered reasonable due to the intrinsic natural variability. In addition, a different extraction temperature was used in this step based on previous data available in the literature (Purohit and Gogate, 2015; Stupar et al., 2021). Regarding the pigment recoveries, in Tables 2.3-2.5 the amounts of total carotenoids spectrophotometrically assessed are reported for all the investigated extraction processes. Conversely, β-carotene and lutein yields are not reported in all cases, because in some extracts these moieties were detectable but not quantifiable by HPLC analysis (e.g. yellow pepper and pumpkin peels).

Table 2.3. Experimental design and responses of the dependent variables expressed as mg/mL of extract when working on carrot peels and yellow pepper peels using HDES 6.

Experiment No.	HBA:HBD Molar ratio	Solvent:sample ratio (v/w)	Extraction time (min)	Carrot Peels			Yellow Pepper Peels	
				Total Carotenoids (mg/mL)	β-Carotene (mg/mL)	Lutein (mg/mL)	Total Carotenoids (mg/mL)	Lutein (mg/mL)
1	0.25	50	60	3.230 ± 0.030	3.135 ± 0.021	0.128 ± 0.012	0.549 ± 0.022	0.505 ± 0.031
2	3	30	60	1.713	1.650	0.042	0.285	0.180
3	3	30	60	1.875	1.160	0.044	0.241	0.220
4	3	10	30	1.340 ± 0.012	1.165 ± 0.064	0.015 ± 0.003	0.159 ± 0.008	0.115 ± 0.016
5	3	50	90	1.240 ± 0.234	0.980 ± 0.156	0.110 ± 0.009	0.352 ± 0.022	0.250 ± 0.004
6	3	10	90	0.266 ± 0.009	0.115 ± 0.007	0.010 ± 0.002	0.077 ± 0.007	0.035 ± 0.007
7	5.75	30	90	0.771 ± 0.530	0.585 ± 0.035	0.069 ± 0.013	0.180 ± 0.004	0.155 ± 0.012
8	0.25	30	90	0.915 ± 0.530	0.820 ± 0.085	0.061 ± 0.046	0.252 ± 0.011	0.180 ± 0.009
9	3	30	60	1.773	1.170	0.038	0.272	0.160
10	3	30	60	1.677	1.230	0.044	0.271	0.210
11	5.75	10	60	0.525 ± 0.099	0.395 ± 0.021	0.010 ± 0.004	0.067 ± 0.008	0.015 ± 0.001
12	5.75	50	60	3.210 ± 0.181	2.940 ± 0.099	0.137 ± 0.003	0.311 ± 0.005	0.280 ± 0.023
13	3	30	60	1.452	1.190	0.039	0.271	0.150
14	5.75	30	30	2.553 ± 0.011	2.250 ± 0.028	0.049 ± 0.004	0.183 ± 0.005	0.255 ± 0.005
15	3	50	30	3.835 ± 0.089	3.400 ± 0.354	0.117 ± 0.026	0.416 ± 0.012	0.375 ± 0.001
16	0.25	30	30	4.359 ± 0.012	4.095 ± 0.120	0.097 ± 0.001	0.401 ± 0.003	0.370 ± 0.023
17	0.25	10	60	2.735 ± 0.053	2.320 ± 0.071	0.018 ± 0.005	0.177 ± 0.002	0.130 ± 0.011

Table 2.4. Experimental design and responses of the dependent variables expressed as mg/mL of extract when working on red pepper peels using HDES 9.

Experiment No.	HBA:HBD Molar ratio	Solvent:sample ratio (v/w)	Extraction time (min)	Total carotenoids (mg/mL)	β-Carotene (mg/mL)	Lutein (mg/mL)
1	0.25	50	60	1.600 ± 0.014	0.478 ± 0.530	0.233 ± 0.128
2	3	30	60	1.676	0.820	0.520
3	3	30	60	1.351	0.594	0.036
4	3	10	30	2.290 ± 0.064	0.445 ± 0.082	0.885 ± 0.044
5	3	50	90	2.268 ± 0.161	0.591 ± 0.046	0.864 ± 0.068
6	3	10	90	1.863 ± 0.743	0.485 ± 0.150	0.048 ± 0.012
7	5.75	30	90	1.428 ± 0.099	0.587 ± 0.078	0.140 ± 0.088
8	0.25	30	90	1.450 ± 0.087	0.459 ± 0.036	0.246 ± 0.125
9	3	30	60	2.003	0.375	0.585
10	3	30	60	2.551	0.627	0.819
11	5.75	10	60	1.863 ± 0.212	0.580 ± 0.012	0.375 ± 0.057
12	5.75	50	60	2.574 ± 0.161	0.575 ± 0.046	0.891 ± 0.068
13	3	30	60	2.665	0.435	1.275
14	5.75	30	30	2.699 ± 0.161	0.521 ± 0.046	0.966 ± 0.068
15	3	50	30	1.990 ± 0.212	0.705 ± 0.011	0.540 ± 0.036
16	0.25	30	30	1.648 ± 0.068	0.563 ± 0.034	0.098 ± 0.021
17	0.25	10	60	2.598 ± 0.161	0.636 ± 0.046	0.981 ± 0.068

Table 2.5. Experimental design and responses of the dependent variables expressed as mg/mL of extract when working on pumpkin peels using HDES 2.

Experiment No.	HBA:HBD Molar ratio	Solvent:sample ratio (v/w)	Extraction time (min)	Total carotenoids (mg/mL)	β-carotene (mg/mL)
1	0.25	50	60	0.430 ± 0.236	0.277 ± 0.091
2	3	30	60	1.430	0.870
3	3	30	60	0.760	0.235
4	3	10	30	2.265 ± 0.211	0.859 ± 0.012
5	3	50	90	1.365 ± 0.017	0.528 ± 0.009
6	3	10	90	1.565 ± 0.016	0.957 ± 0.021
7	5.75	30	90	1.445 ± 0.087	0.586 ± 0.009
8	0.25	30	90	1.740 ± 0.112	0.215 ± 0.016
9	3	30	60	0.550	0.426
10	3	30	60	2.705	0.975
11	5.75	10	60	0.820 ± 0.021	0.152 ± 0.045
12	5.75	50	60	0.860 ± 0.236	0.186 ± 0.091
13	3	30	60	2.895	0.071
14	5.75	30	30	0.281 ± 0.236	0.253 ± 0.091
15	3	50	30	0.290 ± 0.049	0.088 ± 0.004
16	0.25	30	30	2.485 ± 0.149	1.605 ± 0.067
17	0.25	10	60	1.320 ± 0.092	0.590 ± 0.013

2.2.3.1. Models Fitting and Statistical Verification

The experimental data reported in Tables 2.3, 2.4, and 2.5 were statistically elaborated, and the determination coefficients (R^2 and R^2_{adj}), the linear and quadratic effects of the factors, as well as their interaction, the lack of fit, and the significance (p value) of the models for each response variable are summarized in Tables 2.6 and 2.7. The obtained data revealed that the calculated mathematical models showed a good fit ($R^2 > 0.70$) for all the responses for each matrix. Specifically, concerning the extraction from each matrix, the calculated models explained 98.10, 98.74, and 98.65 % of the results for the total carotenoids, β-carotene and lutein yields for the carrot peels, respectively; 97.77 and 94.66 % for total carotenoids and lutein for the extraction from yellow pepper peels, respectively; 93.27, 83.97, and 97.10 % for total carotenoids, β-carotene and lutein in the case of red and yellow pepper peels, respectively; 88.83 and 96.31 % for total carotenoids and β-carotene when extracting the bioactive compounds from pumpkin peels, respectively.

Furthermore, the models' validity in fitting the experimental data was also corroborated by the lack-of-fit *p* values for the different equation models, which were all not significant ($p > 0.05$).

Table 2.6. Coded second-order regression coefficients, determination coefficients (R^2 and R^2_{adj}), lack of fit, and p values of the fitted models on the investigated responses for carrot and yellow pepper peels.

		Carrot Peels			Yellow Pepper Peels	
		Total Carotenoids (mg/mL)	β-carotene (mg/mL)	Lutein (mg/mL)	Total Carotenoids (mg/mL)	Lutein (mg/mL)
Constant	β_0	4.297 ***	4.354 ***	$9.456 \cdot 10^{-2}$ ***	$1.893 \cdot 10^{-1}$ ***	$2.281 \cdot 10^{-1}$ ***
	β_1	-1.269 ***	-1.291 ***	$-2.927 \cdot 10^{-2}$	$-4.239 \cdot 10^{-2}$ ***	$-6.076 \cdot 10^{-2}$ **
Linear	β_2	$3.114 \cdot 10^{-2}$ ***	$2.141 \cdot 10^{-2}$ ***	$5.958 \cdot 10^{-4}$ ***	$8.127 \cdot 10^{-3}$ ***	$9.424 \cdot 10^{-3}$ ***
	β_3	$-1.294 \cdot 10^{-2}$ ***	$-2.422 \cdot 10^{-2}$ ***	$-1.778 \cdot 10^{-3}$	$-2.046 \cdot 10^{-4}$ ***	$-3.476 \cdot 10^{-3}$ **
	β_{11}	$7.975 \cdot 10^{-2}$ *	$9.521 \cdot 10^{-2}$ *	$2.525 \cdot 10^{-3}$ *	$7.180 \cdot 10^{-4}$	$6.257 \cdot 10^{-3}$
Quadratic	β_{22}	$3.097 \cdot 10^{-4}$	$4.938 \cdot 10^{-4}$	$3.246 \cdot 10^{-5}$ **	$5.660 \cdot 10^{-6}$	$2.671 \cdot 10^{-6}$
	β_{33}	$-1.685 \cdot 10^{-4}$	$-6.944 \cdot 10^{-5}$	$9.766 \cdot 10^{-6}$ *	$-2.165 \cdot 10^{-5}$	$9.646 \cdot 10^{-6}$
	β_{12}	$9.955 \cdot 10^{-3}$ **	$7.864 \cdot 10^{-3}$ *	$7.619 \cdot 10^{-5}$	$-5.840 \cdot 10^{-5}$ **	$-4.979 \cdot 10^{-4}$
Interaction	β_{13}	$5.036 \cdot 10^{-3}$ *	$4.879 \cdot 10^{-3}$ *	$1.681 \cdot 10^{-4}$ **	$4.435 \cdot 10^{-4}$ **	$2.727 \cdot 10^{-4}$
	β_{23}	$-6.338 \cdot 10^{-4}$ *	$-5.708 \cdot 10^{-4}$ *	$-6.332 \cdot 10^{-7}$	$7.688 \cdot 10^{-6}$	$-1.875 \cdot 10^{-5}$
R^2		0.9810	0.9874	0.9865	0.9777	0.9466
R^2_{adj}		0.9566	0.9507	0.9690	0.9902	0.8780
Lack of Fit		0.0934	0.2384	0.1128	0.3135	0.1383
p value		< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0011

Table 2.7. Coded second-order regression coefficients, determination coefficients (R^2 and R^2_{adj}), lack of fit, and p values of the fitted models on the investigated responses for red pepper and pumpkin peels.

		Red Pepper Peels			Pumpkin Peels	
		Total Carotenoids (mg/mL)	β-carotene (mg/mL)	Lutein (mg/mL)	Total Carotenoids (mg/mL)	β-carotene (mg/mL)
Constant	β_0	$4.684 \cdot 10^{-1}$ ***	$2.191 \cdot 10^{-1}$ ***	$-1.199 \cdot 10^{-1}$ ***	7.744***	$3.471 \cdot 10^{-1}$ ***
Linear	β_1	$6.130 \cdot 10^{-1}$	$-1.592 \cdot 10^{-1}$ *	$1.543 \cdot 10^{-1}$ *	$-5.905 \cdot 10^{-1}$ *	$-2.043 \cdot 10^{-1}$ **
	β_2	$2.379 \cdot 10^{-2}$ *	$2.229 \cdot 10^{-2}$	$2.932 \cdot 10^{-2}$ ***	$-9.674 \cdot 10^{-2}$	$3.105 \cdot 10^{-2}$ ***
	β_3	$3.581 \cdot 10^{-3}$	$6.916 \cdot 10^{-3}$	$4.339 \cdot 10^{-2}$	$-1.312 \cdot 10^{-1}$ *	$-9.719 \cdot 10^{-3}$
Quadratic	β_{11}	$-4.919 \cdot 10^{-1}$ ***	$5.380 \cdot 10^{-3}$	$-2.335 \cdot 10^{-1}$ **	$5.227 \cdot 10^{-2}$ **	$2.651 \cdot 10^{-2}$ ***
	β_{22}	$-5.111 \cdot 10^{-4}$	$-1.594 \cdot 10^{-4}$	$-3.218 \cdot 10^{-4}$ *	$7.688 \cdot 10^{-4}$	$1.557 \cdot 10^{-4}$
	β_{33}	$-4.258 \cdot 10^{-4}$ **	$-6.578 \cdot 10^{-5}$	$-4.128 \cdot 10^{-4}$ ***	$7.042 \cdot 10^{-4}$ *	$9.842 \cdot 10^{-5}$
Interaction	β_{12}	$1.451 \cdot 10^{-2}$ *	$-6.175 \cdot 10^{-3}$ **	$1.060 \cdot 10^{-2}$ **	$-4.500 \cdot 10^{-4}$	$-2.236 \cdot 10^{-3}$ *
	β_{13}	$8.767 \cdot 10^{-3}$ *	$1.935 \cdot 10^{-3}$	$5.275 \cdot 10^{-3}$ **	$8.000 \cdot 10^{-4}$	$1.307 \cdot 10^{-4}$
	β_{23}	$-3.000 \cdot 10^{-5}$	$-6.167 \cdot 10^{-5}$	$-1.458 \cdot 10^{-1}$	$9.833 \cdot 10^{-4}$ *	$-1.599 \cdot 10^{-4}$
R^2		0.9327	0.8397	0.9710	0.8883	0.9631
R^2_{adj}		0.8461	0.6355	0.9337	0.7447	0.9158
Lack of Fit		0.3019	0.1322	0.1237	0.0564	0.1711
p value		0.0024	0.0387	0.0001	0.0126	0.0003

*, **, *** significantly different at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. β_0: constant; β_1: coefficient of the linear effect of HBA:HBD molar ratio; β_2: coefficient of the linear effect of solvent:sample ratio; β_3: coefficient of the linear effect of extraction time; β_{11}: coefficient of the quadratic effect of HBA:HBD molar ratio; β_{22}: coefficient of the quadratic effect of solvent:sample ratio; β_{33}: coefficient of the quadratic effect of extraction time; β_{12}: interaction coefficient of HBA:HBD molar ratio and solvent:sample ratio; β_{13}: interaction coefficient of HBD:HBA molar ratio and extraction time; β_{23}: interaction coefficient of solvent:sample ratio and extraction time.

On this basis, the subsequent elaboration step allowed obtaining second-order polynomial regression equations for each dependent variable by including all the independent variables and their quadratic combinations and removing the variables that had no significance ($p>0.05$). Considering X_1 the HBA:HBD molar ratio, X_2 the solvent:sample ratio and X_3 the extraction time, the final equations for total carotenoids, β-carotene, and lutein yields for each extraction process were coded as follows (Eq. 2.5 – 2.14):

Extraction from carrot peels

$$Total\ Carotenoids = 4.297 + 3.144 \cdot 10^{-2} X_2 - 1.294 \cdot 10^{-2} X_3 + 7.975 \cdot 10^{-2} X_1^2 + 9.955 \cdot 10^{-3} X_1X_2 + 5.036 \cdot 10^{-3} X_1X_3 - 6.388 \cdot 10^{-4} X_2X_3 \quad Eq.(2.5)$$

$$\beta - carotene = 4.354 - 1.291 X_1 + 2.141 \cdot 10^{-2} X_2 - 2.422 \cdot 10^{-2} X_3 + 9.521 \cdot 10^{-2} X_1^2 + 7.864 \cdot 10^{-3} X_1X_2 + 4.789 \cdot 10^{-3} X_1X_3 - 5.708 \cdot 10^{-4} X_2X_3 \quad Eq.(2.6)$$

$$Lutein = 9.456 + 5.958 \cdot 10^{-4} X_2 + 2.525 \cdot 10^{-3} X_1^2 + 3.246 \cdot 10^{-5} X_2^2 + 9.766 \cdot 10^{-6} X_3^2 + 1.681 \cdot 10^{-4} X_1X_3 \quad Eq.(2.7)$$

Extraction from yellow pepper peels

$$Total\ Carotenoids = 1.893 \cdot 10^{-1} - 4.239 \cdot 10^{-2} X_1 + 8.127 \cdot 10^{-3} X_2 - 2.046 \cdot 10^{-4} X_3 - 5.840 \cdot 10^{-5} X_1X_2 + 4.435 \cdot 10^{-4} X_1X_3 \quad Eq.(2.8)$$

$$Lutein = 2.281 \cdot 10^{-1} - 6.076 \cdot 10^{-2} X_1 + 9.424 \cdot 10^{-3} X_2 - 3.476 \cdot 10^{-3} X_3 \quad Eq.(2.9)$$

Extraction from red pepper peels

$$Total\ Carotenoids = 4.684 \cdot 10^{-1} + 2.379 \cdot 10^{-2} X_2 - 4.919 \cdot 10^{-1} X_1^2 - 4.258 \cdot 10^{-4} X_3^2 + 1.451 \cdot 10^{-2} X_1X_2 + 8.767 \cdot 10^{-3} X_1X_3 \quad Eq.(2.10)$$

$$\beta - carotene = 2.191 \cdot 10^{-1} - 1.592 \cdot 10^{-1} X_1 - 6.175 \cdot 10^{-3} X_1X_2 \quad Eq.(2.11)$$

$$Lutein = -1.199 \cdot 10^{-1} + 1.543 \cdot 10^{-1} X_1 + 2.932 \cdot 10^{-2} X_2 - 2.335 \cdot 10^{-1} X_1^2 - 3.218 \cdot 10^{-4} X_2^2 - 4.128 \cdot 10^{-4} X_3^2 + 1.060 \cdot 10^{-2} X_1X_2 + 5.275 \cdot 10^{-3} X_1X_3 \quad Eq.(2.12)$$

Extraction from pumpkin peels

$$Total\ Carotenoids = 7.744 - 5.905 \cdot 10^{-1} X_1 - 1.312 \cdot 10^{-1} X_3 + 5.227 \cdot 10^{-2} X_1^2 + 7.042 \cdot 10^{-4} X_3^2 + 9.833 \cdot 10^{-4} X_2X_3 \quad Eq.(2.13)$$

$$\beta - carotene = 3.471 \cdot 10^{-1} - 2.043 \cdot 10^{-1} X_1 + 3.105 \cdot 10^{-2} X_2 + 2.051 \cdot 10^{-2} X_1^2 - 2.236 \cdot 10^{-3} X_1X_2 \quad Eq.(2.14)$$

2.3.3.2. Response surface plots analysis

Surface plots are helpful tools to visualize the main effect and interaction effects of two or more independent variables (Tolve et al., 2021). In this study, 3D surface response plots were obtained according to the quadratic polynomial model equations (Eq. 2.5 – 2.14). The plots, created as a function of two independent variables at a time, maintaining the third one at its optimized level, are shown in Figure 2.4 (a-c), Figure 2.5 (a-b), Figure 2.6 (a-c), and Figure 2.7 (a-b) for the peels of carrot, yellow and red pepper, and pumpkin, respectively.

As far as the responses related to the extraction from carrot peels, using thymol/DL-menthol HDES 6 (Fig. 2.4 a-c) all surface plots converged in indicating that higher β-carotene, lutein yields, and total carotenoids content values could be obtained when using a solvent having the lowest HBA:HBD molar ratio (1:4). This observation is in contrast to the data acquired in the screening step, in which HDES 7 (thymol/DL-menthol 1:2) performed slightly worse than HDES 6 (thymol/DL-menthol 1:1), even if no significant differences could be highlighted. However, the results obtained in this step suggest that moving towards extreme low values of HBA:HBD molar ratio enhances the solvent extraction capability. As far as time and solvent:sample ratio, the level of these dependent variables affected the responses in different ways. In the first case, as can be observed from the plots, a decrease in the response values was observed at high extraction times. Conversely, as can be expected when considering the solvent:sample ratio, low levels of this variable resulted in lower response values, causing a decrease of the extraction efficiency.

The surface plots of lutein and total carotenoid responses referring to the extraction from yellow pepper peels using the same selected solvent thymol/DL-menthol HDES 6 (Fig. 2.5 a-b) confirmed that low values of HBA:HBD maximize the extraction efficiency in the investigated range of operating conditions. Furthermore, as shown in the plots, this effect is more noticeable regarding the total carotenoids parameter (Fig. 2.5 b) than lutein yield (Fig. 2.5 a). For the variable extraction time, even in this case, high values are not suitable, probably because of the carotenoid degradation occurring when treating the sample for long time at the set temperature (50 °C) (Stupar et al., 2021). Concerning the solvent:sample parameter, for both responses, extremely low values adversely affect the extraction recovery. On the basis of the experimental results, the solvent:sample ratio of 29.763 was identified as the optimal value for maximizing the responses without excessively increasing the process costs.

For the extraction from red pepper peels using thymol/decanoic acid (HDES 9), despite showing very different trends, the surface plots (Fig. 2.6 a-c) converged in identifying as optimal the medium-high level of the solvent:sample ratio for all the responses. As far as the other two independent variables, all responses were maximized for medium-low levels of HBA:HBD molar ratio and extraction temperature.

Lastly, considering the surface plots obtained by the equations 12-13 for the extraction from pumpkin peels using DL-menthol/lactic acid (HDES 2), the trends were very similar, except for the plots representing the surface visualizing the effect of time and solvent:sample ratio for β-carotene, for which high values of solvent:sample ratio maximized the response. Conversely, the variation of the extraction time did not influence the recovery in any way. A different trend can be reported for total carotenoids since a shorter process period was preferable for this response. In addition, low HBA:HBD molar ratio values are related to higher carotenoids recoveries, especially considering the response for the variable total carotenoids.

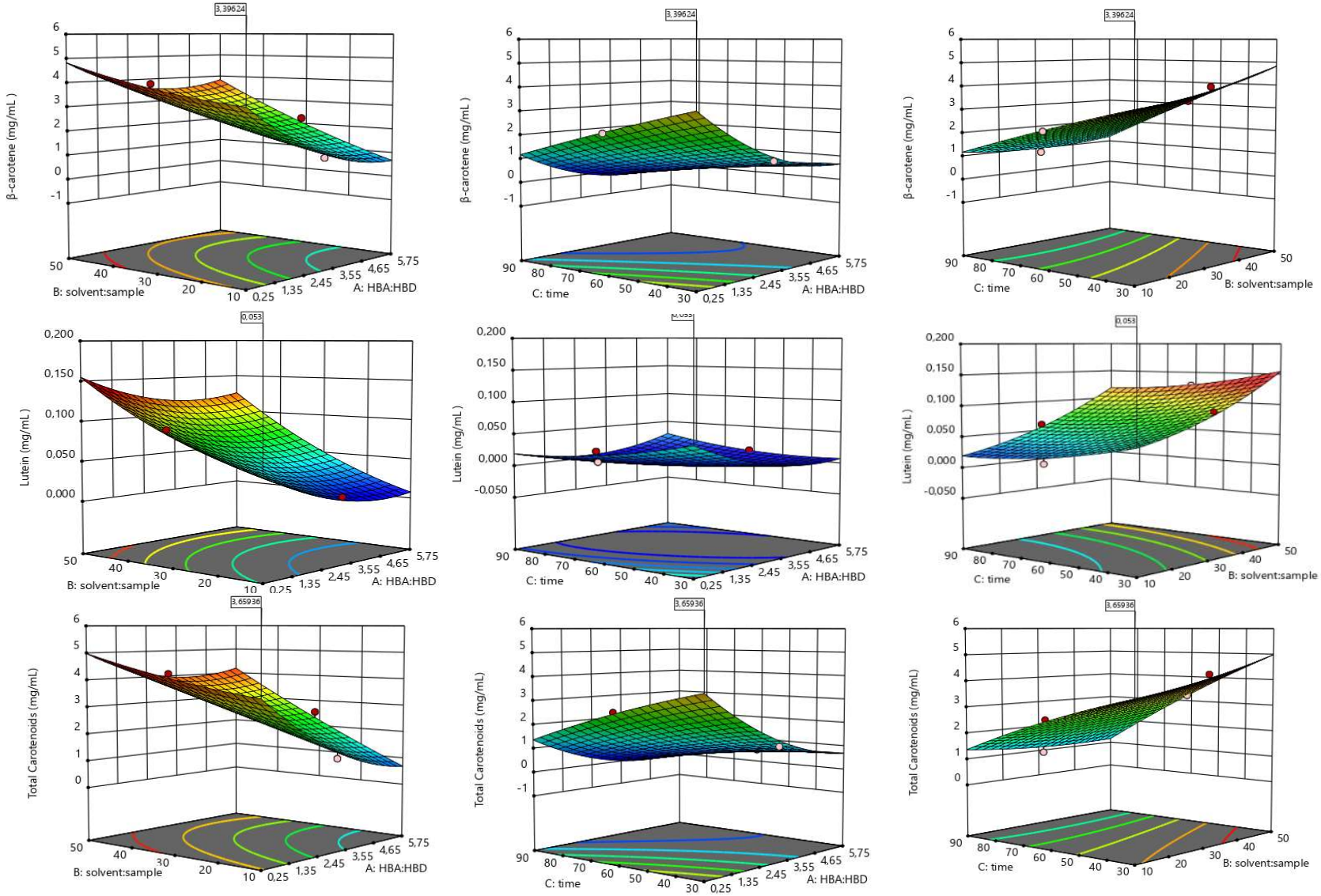


Figure 2.4. Surface Plots of the fitted polynomial equations (Eq. 2.5-2.7) for the responses of the extraction from carrot peels.

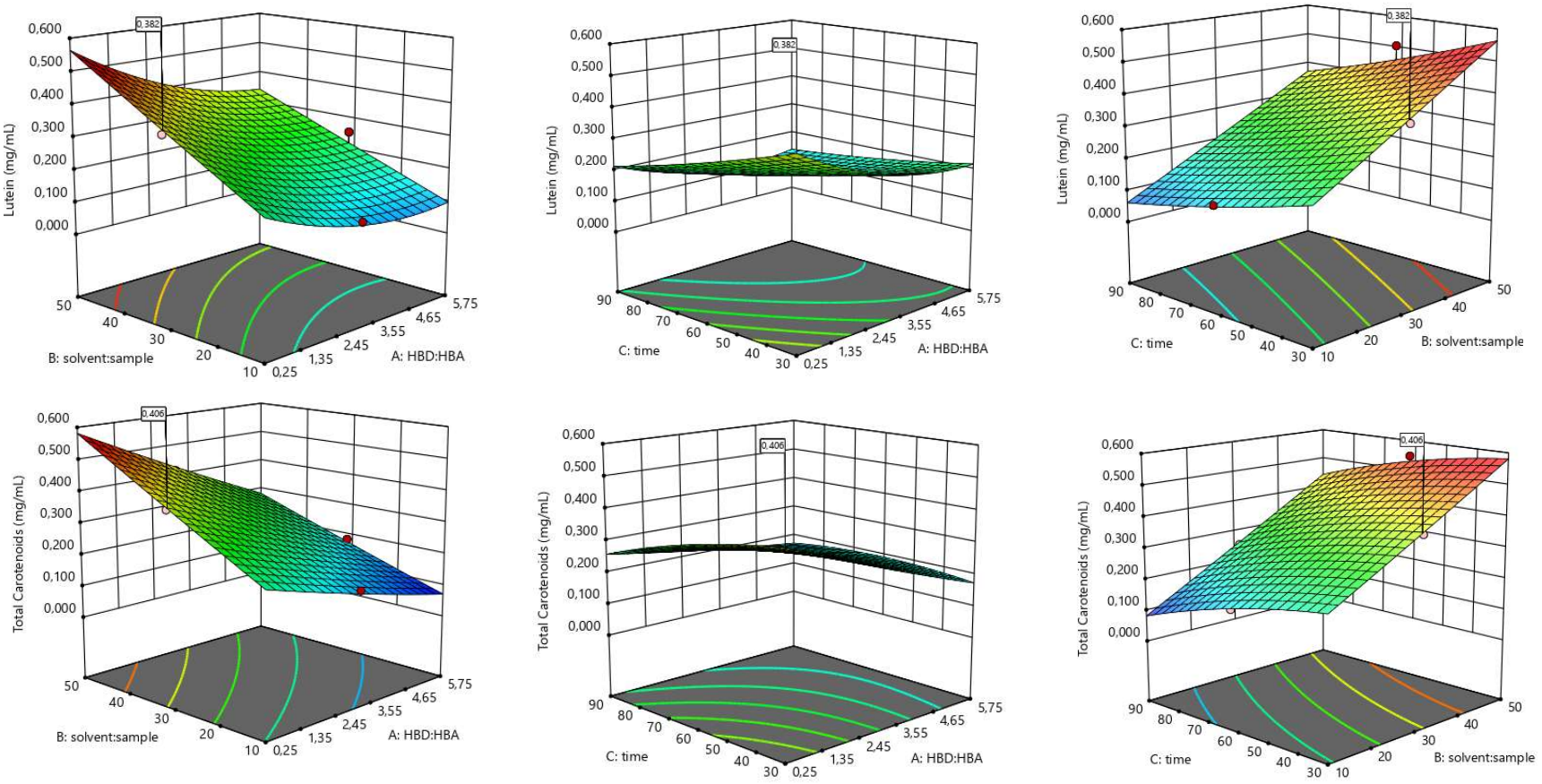


Figure 2.5. Surface Plots of the fitted polynomial equations (Eq. 2.8-2.9) for the responses of the extraction from yellow pepper peels.

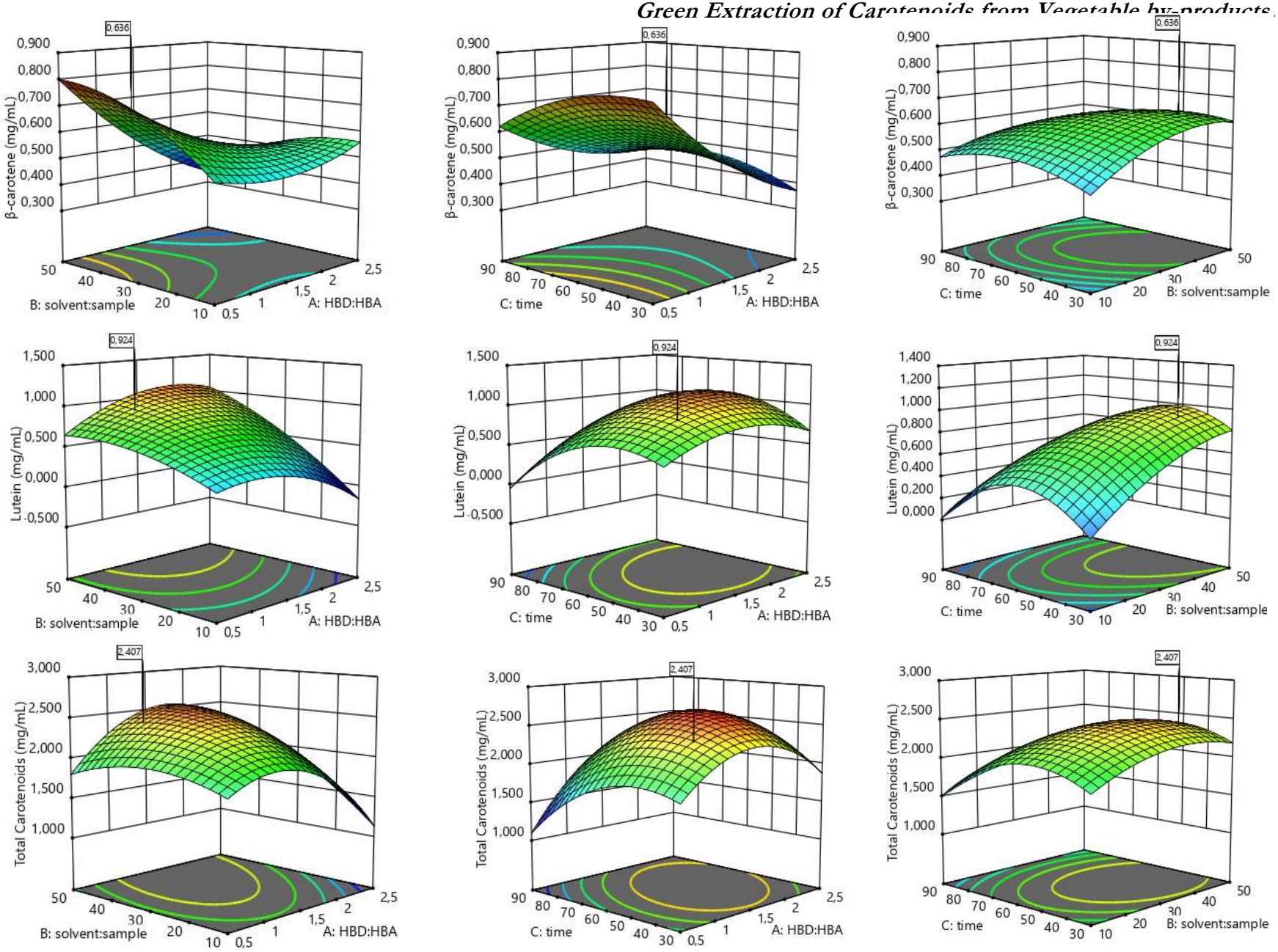


Figure 2.6. Surface Plots of the fitted polynomial equations (Eq. 2.10-2.12) for the responses of the extraction from red pepper peels.

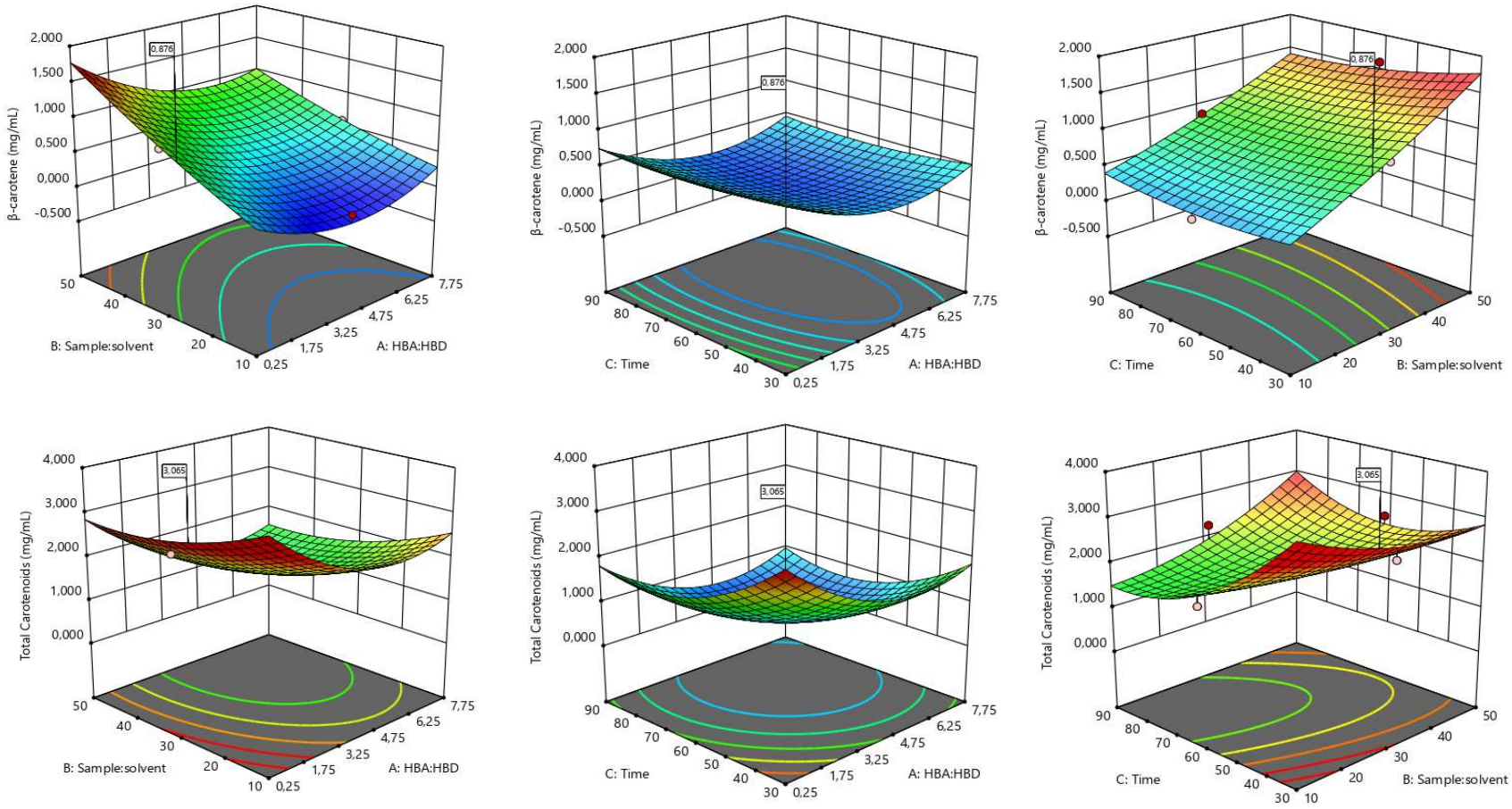


Figure 2.7. Surface Plots of the fitted polynomial equations (Eq. 2.13-2.14) for the responses of the extraction from pumpkin peels.

2.3.3.3. Validation of the predicted models

Utilizing the response data for the total carotenoids, β-carotene and lutein, in conjunction with the model parameters determined with the BBDs, the maximization of all the dependent responses has been carried out using the desirability function, with values ranging from 0 (completely undesirable response) and 1 (fully desirable response). Actually, these parameters were identified applying the software's constraint function 'minimize' to the independent variable solvent to sample ratio and extraction time or both of them to obtain the most favorable situation for optimizing the extraction processes. This was made taking into account the desirability value, which should be as near as possible to 1. The identified optimal values for the extraction processes were as follows:

- HBA:HBD molar ratio equal to 0.25 (thymol/DL-menthol 1:4), solvent to sample ratio 10 and extraction time 30 minutes, with the desirability at 0.91, by minimizing both the variables X_2 and X_3, for carrot peels. As reported above, high solvent:sample ratio values are preferable. However, considering the substantial economic advantages obtainable when a solvent:sample ratio of 10:1 is utilized, especially at the industrial level, the limited decrease in yield was considered an acceptable compromise.
- HBA:HBD molar ratio equal to 0.25 (thymol/DL-menthol 1:4), solvent to sample ratio 29.75 and extraction time 30 minutes, with the desirability at 0.72, by minimizing both the variables X_2 and X_3, for yellow pepper peels.
- HBA:HBD molar ratio equal to 1 (thymol/decanoic acid 1:1), solvent to sample ratio 42.98 and extraction time 41.80 minutes, with the desirability at 0.72, by minimizing only the variable X_3, for red pepper peels;
- HBA:HBD molar ratio equal to 0.25 (DL-menthol/lactic acid 1:4), solvent to sample ratio 26.21 and extraction time 30 minutes, with the desirability at 0.75, by minimizing both the variables X_2 and X_3, for pumpkin peels.

The models' validation was obtained by carrying out the extractions with the identified settings, and the results of the dependent variables are reported in Table 2.8, along with the values predicted by each specific equation and the percentage of Fit. This latter parameter was found to be very high (> 90 %) for all the investigated responses, confirming the very good fit of the selected models for analyzing the experimental data. In addition, with reference to the theoretical maximum yield, the optimized extractions allowed to obtain 30.11, 35.69, and 14.85 % for total carotenoids, β-carotene, and lutein respectively in the extraction from carrot peels; 7.87 and 5.71 % for total carotenoids and lutein considering the yellow

pepper peels; 38.49, 13.70, and 24.88 % for total carotenoids, β-carotene, and lutein from red pepper peels, and 64.41 and 23.50 % for total carotenoids and β-carotene in the extraction from pumpkin peels.

Table 2.8. Predicted and actual experimental values of the investigated responses under the optimal extraction conditions for each investigated matrix.

		Predicted Value (mg/mL)	Experimental Value* (mg/mL)	% Fit
Carrot Peels	Total Carotenoids	3.659	3.545 ± 0.126	96.884 %
	β-carotene	3.396	3.341 ± 0.023	98.380 %
	Lutein	0.053	0.049 ± 0.001	92.451 %
Yellow Pepper Peels	Total Carotenoids	0.406	0.395 ± 0.035	97.291 %
	Lutein	0.382	0.363 ± 0.042	95.026 %
Red Pepper Peels	Total Carotenoids	2.407	2.295± 0.021	95.347 %
	β-carotene	0.636	0.625± 0.052	98.270 %
	Lutein	0.924	0.836± 0.033	90.476 %
Pumpkin Peels	Total Carotenoids	3.065	2.995± 0.021	97.716 %
	β-carotene	0.876	0.823 ± 0.019	93.950 %

* Results are expressed as the mean value of three independent experiments ± standard deviation.

2.4. Conclusions

The by-products derived from the processing of fresh carrots, red and yellow peppers, and pumpkins are a rich source of carotenoids, which can be successfully extracted using NaHDESs. In this study, eleven NaHDESs were physicochemical characterized and tested for their extraction efficiency when used with the four different matrices. The screening step allowed the selection of the thymol/DL-menthol (1:1) NaHDES for the optimization of the extraction processes carried out on carrot and yellow pepper peels, while for red pepper and pumpkin peels, thymol/decanoic acid NaHDES (3:2) and DL-menthol/lactic acid (1:2) NaHDES resulted to be the most suitable ones, respectively. By implementing the BBD and RSM, the extraction processes optimization was achieved obtaining recoveries of 3.341 ± 0.023 and 0.049 ± 0.001 mg/mL of β-carotene and lutein for carrot peels; 0.363 ± 0.042 mg/mL of lutein for yellow pepper peels; 0.625± 0.052 and 0.836± 0.033 mg/mL of β-carotene and lutein for red pepper peels, and 0.823 ± 0.019 mg/mL for pumpkin peels. These promising results were found to be very similar or better to those obtained using acetone as a solvent.

In conclusion, from a green chemistry perspective, the extraction process using NaHDESs has shown interesting perspectives, relying on solvents having higher extraction efficiency, lower energy requirements, and lower environmental impact. Thus, NaHDESs emerge as an attracting solvent

category, offering a potential alternative for extracting natural compounds, which could find use in various food applications, starting from raw materials, food wastes, or by-products.

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2.6. Supplementary

Table S2.1. Experimental densities of the investigated NaHDESs as function of temperature.

	Density (ϱ, g·cm^3)*						
	20 °C	30 °C	40 °C	50 °C	60 °C	70 °C	80 °C
HDES 1	0.981	0.972	0.964	0.951	0.938	0.933	0.917
HDES 2	1.031	1.021	1.013	1.001	0.998	0.992	0.981
HDES 3	0.898	0.891	0.884	0.878	0.872	0.866	0.861
HDES 4	0.894	0.887	0.883	0.874	0.862	0.845	0.843
HDES 5	0.921	0.912	0.905	0.898	0.893	0.883	0.877
HDES 6	0.935	0.922	0.917	0.907	0.893	0.879	0.875
HDES 7	0.924	0.914	0.906	0.901	0.889	0.875	0.866
HDES 8	n.a.	n.a.	0.939	0.923	0.915	0.905	0.890
HDES 9	0.919	0.911	0.903	0.888	0.879	0.870	0.863
HDES 10	0.931	0.922	0.918	0.906	0.889	0.879	0.871
HDES 11	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

* Results are expressed as the mean value of two independent measurements.
n.a. = not assessable.

Table S2.2. Parameters, a and b of Equation (2.2) and respective correlation coefficient (R^2), describing temperature dependence of density of the investigated NaHDESs.

	a (g·cm^3)	b (g·cm^3·K)	R^2
HDES 1	1.0038	-0.0011	0.9909
HDES 2	1.045	-0.0008	0.9844
HDES 3	0.9100	-0.0006	0.9982
HDES 4	0.9161	-0.0009	0.9625
HDES 5	0.9341	-0.0007	0.9969
HDES 6	0.9560	-0.0010	0.9852
HDES 7	0.9449	-0.010	0.9869
HDES 8	0.9828	-0.0011	0.9848
HDES 9	0.9394	-0.0010	0.9917
HDES 10	0.9550	-0.0011	0.9800
HDES 11	n.a	n.a	n.a.

n.a. = not assessable.

Table S2.3. Experimental viscosities of the investigated NaHDESs as a function of temperature.

	Viscosity (mPa·s)*								
	20 °C	25 °C	30 °C	35 °C	40 °C	45 °C	50 °C	55 °C	60 °C
HDES 1	57.132	55.172	54.152	52.962	50.942	50.102	49.582	48.452	47.492
HDES 2	54.821	52.911	51.801	50.881	50.041	48.931	47.901	46.911	46.251
HDES 3	134.969	133.769	132.759	131.438	130.684	129.274	128.821	127.831	127.045
HDES 4	35.871	34.211	32.600	31.210	30.010	28.938	27.928	27.116	26.454
HDES 5	31.799	29.863	28.152	26.562	25.202	24.572	23.359	22.438	21.739
HDES 6	37.963	36.889	35.178	34.032	33.172	32.158	31.615	30.644	29.645
HDES 7	55.963	53.989	52.878	52.004	50.544	49.602	48.580	47.593	46.667
HDES 8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
HDES 9	18.862	16.998	15.477	14.203	13.243	12.101	11.279	10.492	10.486
HDES 10	25.986	24.012	22.356	21.059	20.244	18.944	18.221	17.230	16.304
HDES 11	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

n.a.= not assessable.

Table S2.4. Parameters, A_η B_η, and C_η of Equation (2.3) and respective correlation coefficient (R^2), describing temperature dependence of viscosity of the investigated NaHDESs.

	Aη (mPa·s)	Bη (mPa·s)	Cη (K)	R^2
HDES 1	1.453	34.240	180.198	0.9809
HDES 2	1.374	58.808	131.052	0.9887
HDES 3	1.99	22.000	129.598	0.9856
HDES 4	1.077	48.861	190.835	0.9923
HDES 5	0.973	46.678	205.0182	0.9912
HDES 6	1.117	62.794	157.609	0.9827
HDES 7	1.313	80.519	107.268	0.9996
HDES 9	1.453	34.240	180.198	0.9812
HDES10	1.374	58.808	131.052	0.9912

3. Green Extraction of Carotenoids from *Chlorella vulgaris* Using Hydrophobic Natural Deep Eutectic Solvents based on Fatty Acids

The content of this chapter was published in:

27th Workshop on *the Developments in the Italian PhD Research on Food Science Technology and Biotechnology,* University of Naples, Portici (2023). **Sportiello, L.** Optimization of the extraction techniques using Natural Hydrophobic Deep Eutectic Solvents for the recovery of biomolecules from food and food industry by-products. Oral Presentation.

3.1. Introduction

The number of studies on microalgae has grown vastly in recent years due to their potential as raw materials for various chemical compounds that can be obtained through primary and secondary treatment (Chun-Yen et al., 2012). Primary metabolites such as lipids, proteins, and carbohydrates can be extracted from microalgae and used for various purposes, ranging from biodiesel generation to food supplements and functional foods production (Shen et al., 2019; Lafarga et al., 2020; Scarponi et al., 2021; Coronado-Reyes et al., 2022). Additionally, microalgae are rich sources of secondary metabolites such as pigments, including chlorophylls, which represent a significant portion of the biomass's dry weight, and carotenoids, which belong to the secondary metabolites group of terpenes. β-carotene is the main carotenoid found in microalgae, associated with lipids, chlorophylls, and thylakoids in chloroplasts. These pigments have been evaluated for antioxidant activity and their extraction has been accomplished with a variety of solvents, from hazardous traditional organic compounds to safer solvents (e.g. ethyl acetate, acetone, ethanol), to unconventional ones (supercritical fluids, vegetable oils), often characterized by high costs (Tirado and Calvo, 2019; Liu et al., 2021). However, there has been limited research on the extraction of pigments using NaHDES (Pitacco et al., 2022; Xu et al., 2022; Fan et al., 2022), and none of the reported studies dealt with the extraction from the microalga *Chlorella vulgaris*. This green alga is a spherically-shaped single-cell organism, with a diameter ranging from 2 to 10 μm (Safi et al., 2014) and a cellulose-based cell wall with variable thickness and composition based on growth conditions (Guardini et al., 2021). From a nutritional standpoint, these microalgae produce high amounts of xanthophylls and carotenes such as carotenes, lutein, and zeaxanthin, under both normal growth conditions and when stimulated to undergo carotenogenesis.

This study aimed to investigate the extraction of these pigments from Chlorella vulgaris biomass, testing seven NaHDESs made up of medium-chain fatty acids. After a physicochemical characterization and a screening of their extraction efficiency, the best extracting NaHDES was selected and used in the optimization step of the ultrasound-assisted extraction by implementing the Box-Benhken Design (BBD) combined with Response Surface Modelling (RSM).

3.2. Material and methods

3.2.1. Standards, reagents, and solvents

Decanoic acid (≥ 98.0 %), dodecanoic acid (≥ 98.0 %), nonanoic acid (≥ 96.0 %), and octanoic acid (= 98 %) were used for NaHDESs preparation. Furthermore, acetone (≥ 99.8 %) was used for the comparative extraction using a traditional solvent. The 2,2-diphenyl-1-picrylhydrazyl, 2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS ≥ 98 %), potassium persulphate (98 %), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox 97 %) were used for the antioxidant assays. Acetonitrile (≥ 99.9 %), triethylamine (≥ 99.8 %), and the standards β-carotene (96.4 %), lutein (95.7 %) and zeaxanthin (97.5 %) were used for the HPLC analysis. All the chemicals were purchased from Merck KGaA (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

3.2.2. Matrix preparation

A mutant strain of *Chlorella vulgaris* microalga was cultivated at 25°C in modified Tris-Acetate-Phosphate medium (Gorman and Lenvine, 1965), buffered with NaOH (pH 6.8), stirred and bubbled with CO_2. Once reached the desired growth level ($1 \cdot 10^7$ cell/mL), the biomass was centrifuged at 4000 g for 5 minutes, washed to remove dissolved salts, and freeze-dried using a LIO-5P DGT lyophilizer (Vetrotecnica, PD, Italy). The lyophilized sample was further homogeneously ground in a Polymix PX-MFC 90D mill (Vetrotecnica, PD, Italy), and the resulting powder was vacuum-sealed and stored at -20 °C.

3.2.3. NaHDESs preparation

Seven NaHDESs were prepared according to the method proposed by Dai et al. (2013), with slight modifications. The HBA and the HBD were combined in a specific molar ratio and mixed at 750 rpm under mild heating at 60 °C until a clear transparent liquid was formed. Then, the resulting solvents were gradually cooled to room temperature, and their stability was monitored during storage.

3.2.4. NaHDESs characterization

The prepared NaHDESs were physicochemically characterized in terms of density and rheological behavior as well as by Fourier Transform-Infrared (FT-IR) Spectroscopy.

3.2.4.1. Density

NaHDESs density was measured utilizing a pycnometer (Merck KGaA, Darmstadt, Germany). The measurements were carried out in the temperature range 20-80 °C. The density temperature dependence was described using the following equation (Eq. 3.1):

$$\rho = a + bT \qquad (Eq. 3.1)$$

Where ϱ is the density in grams per cubic centimeter, T is the temperature in °C, and a and b are the fitting parameters. The experimental density results as a function of temperature and the adjustable parameters (a and b) were determined from the fitting of the experimental density data to Eq. 3.1. and are reported in Table S3.1. and S3.2. respectively, in the Supplementary Section.

3.2.4.2. Viscosity and rheological behavior

The prepared NaHDESs were investigated for their rheological properties using a DSR 500 CP4000 rheometer (Lamy Rheology, Champagne-au-Mont-d'Or, France). In all cases, viscosity measurements were carried out using the measuring system MK-SV418 applying different shear-rates, ranging from 50 to 300 s^{-1}. The viscosity values were fitted to the Power Law model as a function of shear rate as described by Equation 3.2. in order to calculate the flow behavior (n) and flow consistency (K) indices (Macosko, 1994).

$$\eta(\dot{\gamma}) = \mathrm{K} \cdot \dot{\gamma}^{\mathrm{n}-1} \qquad (Eq. 3.2)$$

Where η is the viscosity (mPa·s), *K* is the flow consistency index (mPa·s) and represent the viscosity at the shear rate $\dot{\gamma} = 1\ s^{-1}$ and *n* is the power law index (unitless) defining the steepness of the shear thinning decay for $n < 1$ (Eberhard et al. 2019). Furthermore, the measurements were also conducted in the temperature range 20-60 °C and the obtained values were fitted as a function of temperature, using the Vogel-Fulcher-Tammann (VTF) model:

$$\ln \eta = A_\eta + \frac{B_\eta}{(T - C_\eta)} \qquad (Eq. 3.3)$$

where η is the viscosity in mPa·s, T is the temperature in K, and $A\eta$, $B\eta$, and $C\eta$ are adjustable parameters. The experimental viscosity results as a function of temperature are presented in Table S.3.3 in the Supplementary Section.

3.2.5. NaHDESs screening for the extraction of carotenoids

0.1 g of lyophilized sample was added to 5 mL of each NaHDES (sample:solvent 1:50 w/v), vortexed at 25 °C for 60 seconds, and then kept under continuous mixing for 30 minutes using a disc rotator (UniLOPMIX2, LLG-Labware, Meckenheim, Germany). Afterwards, the mixture was sonicated for 60 minutes at 45 kHz (2200 MH S3, SOLTEC, Milan, Italy) before being centrifuged at 3900 RCF for 10 minutes. All manipulations were carried out away from direct light to minimize the photodecomposition of carotenoids throughout the analytical procedure. The extraction under the same experimental conditions using acetone as the extraction solvent was also carried out to compare the extraction efficiency of the investigated NaHDESs with that of a conventional organic solvent. Furthermore, exhaustive extractions were conducted to compare the extraction efficiency of each solvent to the maximum extraction yield obtainable from *Chlorella vulgaris* biomass. All extractions were performed in triplicate, and the results were expressed as the mean value ± standard deviation.

3.2.6. Extraction efficiency determination

During the screening step, UV-Vis spectrophotometry was used to monitor the extraction efficiency of carotenoids from *Chlorella vulgaris*. In the optimization phase, additional methods were used, such as HPLC-DAD analysis to quantify the yields of β-carotene, lutein, and zeaxanthin, as well as antioxidant activity assessment using ABTS and DPPH assays.

3.2.6.1. UV-Vis spectrophotometry

The carotenoid-rich extracts were diluted with acetone (1:50, v/v) and spectra measurements from 350 to 750 nm were taken using a V-750 UV-Visible Spectrophotometer (Jasco, Oregon, USA). The obtained data were then fitted using the 'solver' add-in of Excel, according with the estimation method proposed by Chazaux et al. (2022) and the content of Total Carotenoids was calculated and expressed as mg/mL of extracts.

3.2.6.2. HPLC-DAD analysis

Carotenoid analysis was carried out according to the method described by Perozeni et al. (2020). Lutein, zeaxanthin, and β-carotene were tentatively identified and quantified using a C18 column (Gemini 3 μm

C18 110 A 50x4,6 mm, Phenomenex) and an LC-4000 system with an MD-4010 PDA detector (Jasco, Oregon, USA). Moiety separation was achieved by gradient elution [A to B from 0 to 100% in 15 min, where A= ethyl acetate, B= acetonitrile–water–triethylamine (9:1:0.01, v/v/v)] at the flow rate of 1.5 mL/min and recording the chromatogram at of 440 nm. Lutein, zeaxanthin, and β-carotene identification was achieved by comparing their retention times and spectra to those obtained utilizing commercially available standards (Extrasynthese, Genay, France). Furthermore, pigment quantification was conducted using the external standard technique.

3.2.6.3. Antioxidant analysis

The antioxidant capacity of the rich-carotenoids extracts was assessed using both 2,2'-azinobis-(3-ethylenebenzothiazoline)-6-sulfonic acid (ABTS) assay and the 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH) assay. The ABTS determination was performed according to the method of Re et al. (1999), with adaptations. After preparation, the $ABTS^{\bullet+}$ stock solution was diluted with H_2O, pH 7.4, to an absorbance of 0.70 (± 0.02) at 734 nm. Subsequently, 9.8 mL of $ABTS^{\bullet+}$ solution was mixed with 200 μL of sample in a 15 mL tube and stirred for 60 minutes at room temperature. The spectrophotometric measurement was performed at 734 nm, and the antioxidant capacity was expressed in millimol Trolox equivalent per milliliter of extract (mmolTE/mL of extract).

The DPPH assay was carried out following the method proposed by Maggini et al. (2018). Thirty μL of extracts were placed in tubes with 2.97 mL of DPPH solution (20 mg/L DPPH in methanol,). After 45 minutes of incubation at room temperature, in dark conditions, and under continuous mixing, the antioxidant power was measured spectrophotometrically at 515 nm. The percentage inhibition of the DPPH radical per milliliter of extract was calculated from the absorbance values of the blank (A_{blank}) and of the sample (A_{sample}) as follows:

$$\%\ \text{Inhibition mL}^{-1} = 100 \cdot [(A_{blank} - A_{sample}) / A_{blank})] / \text{mL}$$

3.2.7. Experimental design for the extraction optimization

The extraction optimization was carried out implementing a BBD coupled with RSM. Preliminary experiments were performed to identify the main factors and their ranges. HBA:HBD molar ratio (X_1), solvent to sample ratio (X_2), and extraction time (X_3) were the most significant variables and were .investigated at three different levels and coded as -1, 0, and +1. Furthermore, the extraction temperature

was fixed at 50 °C, as previously optimized by Stupar et al. (2019). The experimental range and levels of the independent variables are listed in Table 3.1.

Table 3.1. Experimental range with the coded and natural values of the independent variables for carotenoid extraction from *Chlorella vulgaris*.

Independent variables	Levels		
	-1	0	-1
HBA:HBD molar ratio	1	4.5	8
Solvent:sample ratio (v/w)	10	30	50
Extraction time (min)	30	60	90

The total carotenoids and the yields of lutein, β-carotene and zeaxanthin, together with the antioxidant assays ABTS and DPPH were selected as dependent response variables to be optimized in the extraction process. A second-order model was adopted to explain the relationship between the dependent responses and the factors using the following quadratic equation (Equation 3.4):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} x_i x_j + \varepsilon \qquad Eq.(3.4)$$

where Y is the response variable, k is the number of the patterns, i and j are the index numbers for pattern, β_0 is the 'intercept term', x_i, x_j,..., x_k are the coded independent variables, β_i and β_{ii} are respectively the linear and the quadratic coefficients, β_{ij} is the interaction effect, while ε is the random error explaining differences between predicted and measured values. The extraction efficiency of lutein, zeaxanthin, β-carotene and from the vegetable by-products samples was assessed by HPLC-DAD analysis. Design Expert software (Version 8.0.7.1, Stat-Ease Inc., MN, USA) was adopted to set seventeen batch experiments with five central points. To ensure the model strength, the extractive tests, except for the central points, were conducted in triplicate.

3.2.8. Statistical analysis

Statistical analysis of the data obtained during the screening step was performed using the software XLSTAT Premium (Version 2020.3.1, Addinsoft, Paris, France), applying one-way ANOVA. Significant

differences between means were computed by Tukey's HSD (Honestly Significantly Different) test at 95 % confidence interval. In addition, the fitting of equation 2.3 was obtained using the software OriginPro (Version 2023, OriginLab Corporation, Northampton, MA, USA). For the process optimization, the dependent variables were analyzed using the BBD. The final models included terms with a significance level below 0.05 ($p < 0.05$) and those necessary to maintain the hierarchical structure of the model. Multiple linear regression analysis was conducted to create the best-fitted model. The adequacy of the model was evaluated based on the model p-value, lack of fit, coefficient of determination (R^2), and the adjusted coefficient of determination (R^2_{adj}). According to the desirability function, the optimal extraction conditions were calculated to maximize the extraction efficiency of all target responses simultaneously and apply constraints for the variables X_2 (solvent:sample ratio) and X_3 (extraction time) to minimize the process costs. The adequacy of the model was verified by carrying out extractions at the predicted optimal conditions in three replicates. The experimental design, the optimization step, and the construction of three-dimensional response surface plots were obtained using the Design Expert software (Version 8.0.7.1, Stat-Ease Inc., MN, USA).

3.3. Results and discussion

3.3.1. NaHDESs preparation and characterization

In the present investigation, seven different NaHDESs derived from the combination of food-grade medium-chain fatty acids were prepared at specific molar ratios, as reported in Table 3.2. During storage, HDES 17 showed instability when the room temperature dropped at 20 °C, but due to the easy restoration of the liquid state at already 23 °C, the solvent was tested with the others, taking fixed the room temperature at 25 ± 1 °C. Concerning the physicochemical characterization, density, viscosity, and rheological behavior were assessed to acquire valuable information for evaluating the extraction efficiency of the solvents. As for density, the obtained experimental data were found to fit with data reported in the literature (Florindo et al., 2018; Kyriakoudi et al., 2022). The values, ranging from 0.863 to 0.901 g/cm^3 at 25 °C resulted lower than those reported for most hydrophilic DESs (Leron et al., 2012; Xie et al., 2014) and also for those assessed for other hydrophobic DESs (van Osch et al., 2019). The low densities of the starting fatty acids utilized could explain the observed values.

For viscosity, the obtained data, ranged from 8.786 to 15.562 mPa·s at 25 °C, and were generally in agreement with those reported in literature, resulting lower than those assessed for other NaHDESs based on menthol and thymol (Tang et al., 2021; Sportiello et al., 2023). In addition, as reported in the

previous section, NaHDESs viscosity was assessed in the shear rate range 50 – 300 s^{-1} and the results highlighted that the prepared NaHDESs exhibit a shear-thinning behavior in the imposed shear rate range, with a decrease in the viscosity when higher values of shear rate were applied. This statement was validated by the "n" values calculated according to Equation (3.3) (see Section 3.2.4.2). The pertinent data are reported in Table 3.2.

Table 3.2. HBAs/HBDs, molar ratios and physicochemical characterization of the prepared NaHDESs, and carotenoids content of the obtained extracts (mg/mL) as determined by UV-Vis spectrophotometry after extraction with different NaHDESs during the screening step.

NaHDESs	HBA/HBD	Molar Ratio	Density* (g/cm^3 at 25 °C)	Viscosity* (mPa·s at 25 °C)	Flow Behavior Index (n) *	Flow Consistency Index (K) *	Total Carotenoids **, *** (mg/mL)
HDES 12	octanoic acid/decanoic acid	2:1	0.900	9.841	0.648	37.523	1.771 ± 0.069 ^{b}
HDES 13		3:1	0.901	9.642	0.665	34.475	1.675 ± 0.027 ^{bc}
HDES 14		4:1	0.863	8.786	0.809	17.116	1.561 ± 0.001 ^{bcd}
HDES 15	octanoic acid/dodecanoic acid	3:1	0.901	12.591	0.648	51.015	1.550 ± 0.159 ^{bcd}
HDES 16	nonanoic acid/dodecanoic acid	3:1	0.858	15.562	0.639	59.279	2.082 ± 0.019 ^{a}
HDES 17	decanoic acid/dodecanoic acid	2:1	0.892	17.121	0.781	36.216	1.262 ± 0.090 ^{d}
HDES 18	nonanoic acid/octanoic acid/dodecanoic acid	3:1:1	0.896	13.583	0.777	29.343	1.232 ± 0.133 ^{d}
Acetone							1.351 ± 0.035 ^{cd}

* Results are expressed as the mean value of two independent measurements. ** Different lowercase letters as superscripts within the same column for each NaHDES differ significantly according to Tuckey's test at $p < 0.05$. *** Results are expressed as the mean value of three independent experiments ± standard deviation.

Furthermore, as described in the previous sections (3.2.4.1 and 3.2.4.2), density was assessed in the temperature range 20−80 °C and viscosity in the temperature range 20-60 °C. Tabular and graphical data are reported in table S3.1, S3.2, S3.3, S3.4, and in figure 3.1. and 3.2.

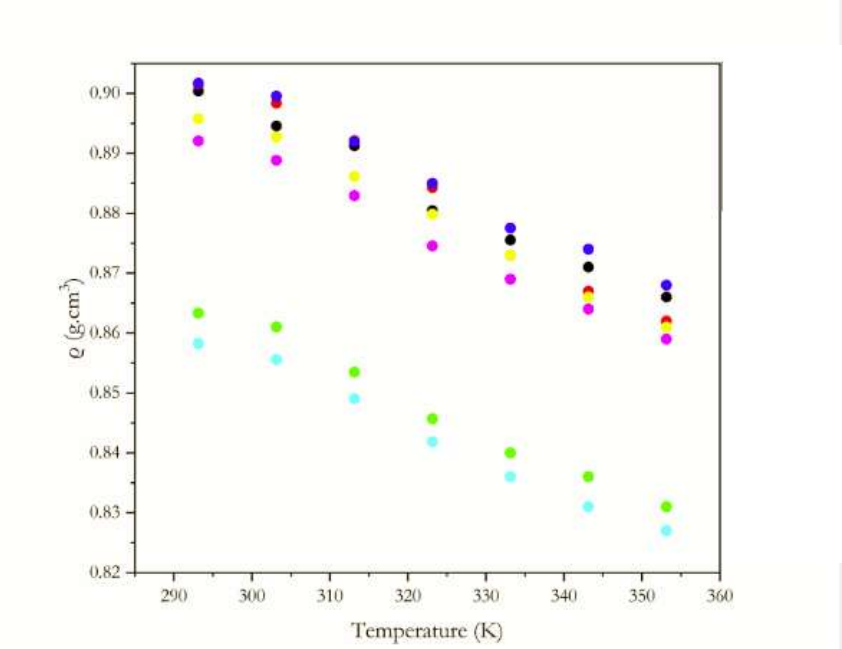


Figure 3.1. Experimental densities of the tested NaHDESs as a function of temperature (black= HDES 12; red= HDES 13; green= HDES 14; blue= HDES 15; light blue= HDES 16; pink= HDES 17; yellow= HDES 18).

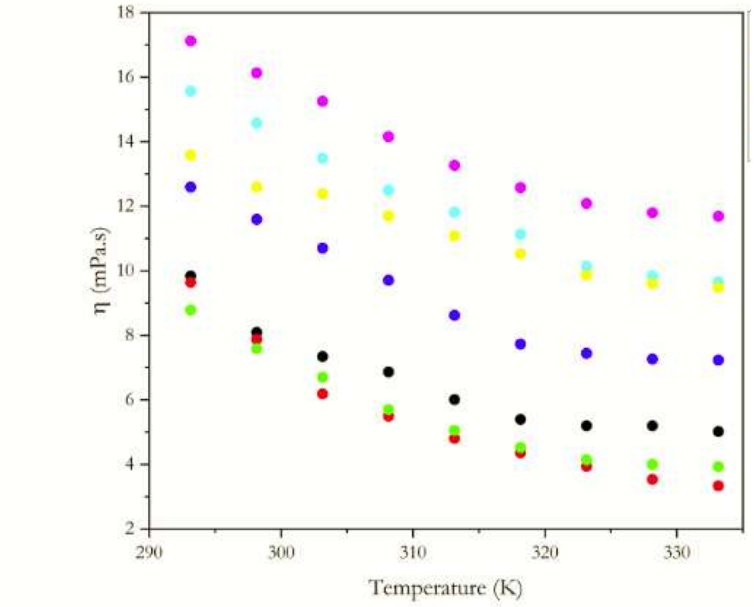


Figure 2.2. Experimental viscosities of the tested NaHDESs as a function of temperature (black= HDES 12; red= HDES 13; green= HDES 14; blue= HDES 15; light blue= HDES 16; pink= HDES 17; yellow= HDES 18).

3.3.2. Selection of the best performing NaHDES

The investigated NaHDESs were tested for their efficiency to extract carotenoids from *Chlorella vulgaris* biomass in order to assess the most efficient solvent and compare the extraction efficiency with that obtained when using the conventional solvent acetone and that available when using the same solvent in an exhaustive extraction. The content of total carotenoids resulted in each extract is reported in Table 3.2. The statistical evaluation of these results was performed using one way ANOVA to identify any significant differences and to select the NaHDESs for further investigation in the optimization step. Five out seven of the tested green solvents allowed to achieve recoveries higher than 100 %, with reference to the acetone extraction, with values ranging from 114.81% (HDES 15) to 154.10 % (HDES 16). For the remaining two, the extraction recoveries were in the range of 91.11-93.33 %. HDES 16, composed by nonanoic and dodecanoic acid at molar ratio 3:1, was found to be the most efficient extracting media for the recovery of carotenoids from the selected biomass, with a percentage of extraction, with reference to the maximum available, of 19.15. The statistical analysis showed that its recovery was significantly different ($p > 0.05$) from all the other solvents, thus was selected for the subsequent experimental step.

3.3.3. Optimization of the extraction process

The optimization of the operating conditions of the extraction process is a crucial step since the associated costs might strongly influence the process scalability at the industrial level. In this case, the scientific approach requires the use of experimental designs, which can be very useful for maximizing the extraction yields, and reducing the number of test trials and resources. In this study, the effect of HBA:HBD molar ratio, solvent sample ratio, and extraction time on the recovered amounts of total carotenoids, lutein, zeaxanthin, and β-carotene, as well as the antioxidant activity, were evaluated using BBD coupled with RSM. The related results are reported in Table 3.3. On average, the obtained data for total carotenoids were lower than that achieved by HDES 16 in the screening phase, except for the extraction test carried out using a HBA:HBD molar ratio of 1:1. This suggested that the level 1 of the variable X_1 could represent the optimal value in the investigated range. With regard to the different carotenoids investigated, the most abundant one resulted to be lutein, followed by β-carotene and zeaxanthin, with relative average percentages of 51.12, 14.10, and 35.96 %, respectively.

As for the antioxidant activity, it is widely recognized that assessing the antioxidant capacity of selected antioxidants requires the utilization of multiple test systems (Deenu et al., 2013). For this reason, the ABTS test was utilized to estimate the free radical-scavenging activity, while the DPPH test was chosen

since this assay finds wide use in food analysis because it is not subject to pH variations and it is useful to analyze both hydrophilic and lipophilic moieties. The obtained results are comparable to the reports present in the literature for this matrix (Kwang et al., 2010; Coronado-Reyes et al., 2020) and ranged from 160.800 ± 49.780 to 1583.300 ± 2.828 mmol TE/mL for ABTS and from 1.305 ± 0.0129 to 33.075 ± 0.212 % of inhibition/mL for DPPH assay.

3.3.3.1. Model fitting and statistical validation

The experimental data reported in Table 3.3 were statistically elaborated, and the determination coefficients (R^2 and R^2_{adj}), the linear and quadratic effects of the factors, as well as their interaction, the lack of fit, and the significance (p value) of the models for each response variable are summarized in Table 3.4. The obtained data revealed that the adopted mathematical model fitted with good determination coefficients ($R^2 > 0.70$) for all the responses. Specifically, the model explained 98.47, 96.56, 94.17, 97.77, 75.42, and 95.24 % of the results for total carotenoids, lutein, zeaxanthin, β-carotene, ABTS, and DPPH, respectively. However, it should be pointed out that the model applied for ABTS response showed insignificant p-value ($p > 0.05$), therefore this dependent variable was excluded from the optimization.

Table 3.3. Experimental design and responses of the dependent variables expressed as mg/mL of the extract for the extraction from *Chlorella vulgaris*.

Experiment No.	HBA:HBD Molar ratio	Solvent: sample ratio (v/w)	Extraction time (min)	Total Carotenoids (mg/mL)	Lutein (mg/mL)	Zeaxanthin (mg/mL)	β-Carotene (mg/mL)	ABTS (mmol TE/mL)	DPPH (% inhibition/mL)
1	4.5	50	30	1.178 ± 0.012	0.581 ± 0.017	0.199 ± 0.074	0.300 ± 0.070	292.000 ± 37.603	18.100 ± 7.283
2	1	30	30	2.124 ± 0.121	0.817 ± 0.079	0.343 ± 0.038	0.541 ± 0.073	736.410 ± 44.675	10.035 ± 0.700
3	4.5	10	30	1.001 ± 0.005	0.877 ± 0.006	0.346 ± 0.049	0.325 ± 0.030	160.800 ± 49.780	2.645 ± 0.271
4	8	30	90	1.454 ± 0.012	0.021 ± 0.065	0.019 ± 0.012	0.030 ± 0.017	464.400 ± 26.850	1.305 ± 0.0129
5	8	10	60	1.002 ± 0.018	0.363 ± 0.124	0.067 ± 0.063	0.185 ± 0.083	266.670 ± 14.103	1.765 ± 0.288
6	4.5	30	60	1.175	0.190	0.022	0.103	794.400	24.240
7	1	50	60	1.867 ± 0.017	0.867 ± 0.057	0.271 ± 0.045	0.499 ± 0.023	616.000 ± 27.357	24.000 ± 0.657
8	1	10	60	1.719 ± 0.126	0.601 ± 0.110	0.197 ± 0.052	0.369 ± 0.058	335.330 ± 2.079	5.250 ± 0.031
9	4.5	30	60	1.175	0.215	0.132	0.150	575.620	31.590
10	4.5	30	60	1.222	0.171	0.044	0.210	673.620	30.330
11	8	50	60	1.263 ± 0.345	0.153 ± 0.050	0.028 ± 0.024	0.080 ± 0.045	1583.300 ± 2.828	20.950 ± 0.091
12	1	30	90	1.916 ± 0.111	0.202 ± 0.017	0.049 ± 0.029	0.125 ± 0.038	625.590 ± 19.936	19.755 ± 0.035
13	4.5	10	90	1.007 ± 0.008	0.152 ± 0.014	0.035 ± 0.012	0.079 ± 0.082	206.000 ± 36.371	4.780 ± 0.179
14	4.5	50	90	1.078 ± 0.021	0.153 ± 0.002	0.068 ± 0.027	0.129 ± 0.030	822.000 ± 42.593	33.075 ± 0.212
15	4.5	30	60	1.777	0.291	0.063	0.150	575.180	31.020
16	8	30	30	1.282 ± 0.118	0.199 ± 0.021	0.056 ± 0.012	0.097 ± 0.027	903.180 ± 19.047	10.740 ± 1.095
17	4.5	30	60	1.275	0.216	0.059	0.080	460.020	25.740

Table 3.4. Coded second-order regression coefficients, determination coefficients (R^2 and R^2_{adj}), lack of fit, and p values of the fitted model on the investigated responses.

		Total Carotenoids (mg/mL)	Lutein (mg/mL)	Zeaxanthin (mg/mL)	β-carotene (mg/mL)	ABTS (mmol Trolox/mL)	DPPH (% inhibition/mL)
Constant	β_0	1.902 ***	1.795 ***	$9.396 \cdot 10^{-1}$ ***	$9.604 \cdot 10^{-1}$ ***	$3.809 \cdot 10^{2}$ ***	$-5.176 \cdot 10^{1}$ ***
	β_1	$-4.415 \cdot 10^{-1}$ **	$-1.286 \cdot 10^{-1}$ ***	$-6.088 \cdot 10^{-2}$ ***	$-1.148 \cdot 10^{-1}$ ***	$-1.974 \cdot 10^{2}$	9.090
Linear	β_2	$4.168 \cdot 10^{-2}$	$-3.207 \cdot 10^{-2}$	$-1.232 \cdot 10^{-2}$	$-9.405 \cdot 10^{-3}$	-1.093 *	1.038 ***
	β_3	$-1.454 \cdot 10^{-3}$	$-1.904 \cdot 10^{-2}$ ***	$-1.316 \cdot 10^{-2}$ *	$-7.426 \cdot 10^{-3}$ ***	$1.153 \cdot 10^{1}$	1.215
	β_{11}	$3.127 \cdot 10^{-2}$ **	$6.057 \cdot 10^{-3}$	$1.291 \cdot 10^{-3}$	$5.498 \cdot 10^{-3}$ *	$1.840 \cdot 10^{1}$	$-8.075 \cdot 10^{-1}$ **
Quadratic	β_{22}	$-6.123 \cdot 10^{-4}$ *	$5.132 \cdot 10^{-4}$ ***	$1.522 \cdot 10^{-4}$	$1.935 \cdot 10^{-4}$ ***	$-2.170 \cdot 10^{-1}$	$-1.425 \cdot 10^{-2}$ *
	β_{33}	$-1.527 \cdot 10^{-5}$	$2.120 \cdot 10^{-6}$	$4.120 \cdot 10^{-6}$	$-8.328 \cdot 10^{-6}$	$-1.164 \cdot 10^{-1}$	$-9.148 \cdot 10^{-3}$ **
	β_{12}	$4.027 \cdot 10^{-4}$	$-1.696 \cdot 10^{-3}$	$-4.008 \cdot 10^{-4}$	$-8.395 \cdot 10^{-4}$ *	3.700	$1.554 \cdot 10^{-3}$
Interaction	β_{13}	$9.045 \cdot 10^{-4}$	$1.040 \cdot 10^{-3}$ **	$6.107 \cdot 10^{-4}$ *	$8.280 \cdot 10^{-4}$ **	$-7.809 \cdot 10^{-1}$	$-4.561 \cdot 10^{-2}$ *
	β_{23}	$-4.416 \cdot 10^{-5}$	$1.236 \cdot 10^{-4}$	$7.512 \cdot 10^{-6}$	$3.135 \cdot 10^{-5}$	$2.020 \cdot 10^{-1}$	$5.350 \cdot 10^{-3}$
R^2		0.9547	0.9656	0.9417	0.9777	0.7542	0.9524
R^2_{adj}		0.8965	0.9213	0.8668	0.9902	0.4381	0.8911
Lack of Fit		0.7410	0.0652	0.5064	0.3135	0.1055	0.3148
p value		0.0006	0.0003	0.0015	< 0.0001	0.1323	0.0008

*, **, *** significantly different at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. β_0: constant; β_1: coefficient of the linear effect of HBA:HBD molar ratio; β_2: coefficient of the linear effect of solvent:sample ratio; β_3: coefficient of the linear effect of extraction time; β_{11}: coefficient of the quadratic effect of HBA:HBD molar ratio; β_{22}: coefficient of the quadratic effect of solvent:sample ratio; β_{33}: coefficient of the quadratic effect of extraction time; β_{12}: interaction coefficient of HBA:HBD molar ratio and solvent:sample ratio; β_{13}: interaction coefficient of HBD:HBA molar ratio and extraction time; β_{23}: interaction coefficient of solvent:sample ratio and extraction time.

Based on the statistical elaboration, second-order polynomial regression equations for each dependent variable were obtained by incorporating all the independent variables and their quadratic combinations, while removing the variables with no significance ($p > 0.05$). Having coded HBA:HBD molar ratio as X_1, the solvent:sample ratio as X_2, and the extraction time as X_3, the final equations for total carotenoids, lutein, zeaxanthin, β-carotene, and DPPH were as follows (Equation 3.5– 3.9):

$$Total\ Carotenoids = 1.902 - 4.415 \cdot 10^{-1} X_1 + 3.127 \cdot 10^{-2} X_1^2 - 6.123 \cdot 10^{-4} X_2^2 \quad Eq.(3.5)$$

$$Lutein = 1.795 - 1.286 \cdot 10^{-1} X_1 - 1.904 \cdot 10^{-2} X_3 + 5.132 \cdot 10^{-4} X_2^2 + 1.040 \cdot 10^{-3} X_1 X_3 \quad Eq.(3.6)$$

$$Zeaxanthin = 9.396 \cdot 10^{-1} - 6.088 \cdot 10^{-2} X_1 - 1.316 \cdot 10^{-2} X_3 + 6.107 \cdot 10^{-4} X_1 X_3 \quad Eq.(3.7)$$

$$\beta - carotene = 9.604 \cdot 10^{-1} - 1.148 \cdot 10^{-1} X_1 - 7.426 \cdot 10^{-3} X_3 + 5.498 \cdot 10^{-3} X_1^2 + 1.935 \cdot 10^{-4} X_2^2 - 8.395 \cdot 10^{-4} X_1 X_2 + 8.280 \cdot 10^{-4} X_1 X_3 \quad Eq.(3.8)$$

$$DPPH = -5.1760 \cdot 10^{1} + 1.038 X_2 - 8.075 \cdot 10^{-1} X_1^2 - 1.425 \cdot 10^{-2} X_2^2 - 9.148 \cdot 10^{-3} X_3^2 - 4.561 \cdot 10^{-2} X_1 X_3 \quad Eq.(3.9)$$

3.3.3.2. Response surface analysis

The carotenoid extraction from *Chlorella vulgaris* biomass was optimized using the Box-Behnken Design coupled with Response Surface Modelling. The surface plots (representing the interaction effects of two of the select variables, while keeping the third at its optimal value) for the responses total carotenoids, lutein, zeaxanthin, β-carotene, and DPPH are reported in Figure 3.3 (a-e). As far as the total carotenoids and the quantified three pigments (Fig. 3.3 a, b, c, and d), the obtained surface plots converged in indicating a substantially similar effect of the independent variables HBA:HBD molar ratio and extraction time on the responses, since higher values can be observed when the variables were at their lowest levels. Regarding the solvent:sample ratio, the extraction of total carotenoids could be maximized when not working at extreme conditions, while for lutein, zeaxanthin and β-carotene higher values can be obtained when carrying out thr extraction at the low or the high set level. A different trend is depicted for DPPH, because medium-high levels of solvent:sample ratio and extraction time, along with medium level of HBA:HBD molar ratio, might allow to exhibit higher antioxidant properrties.

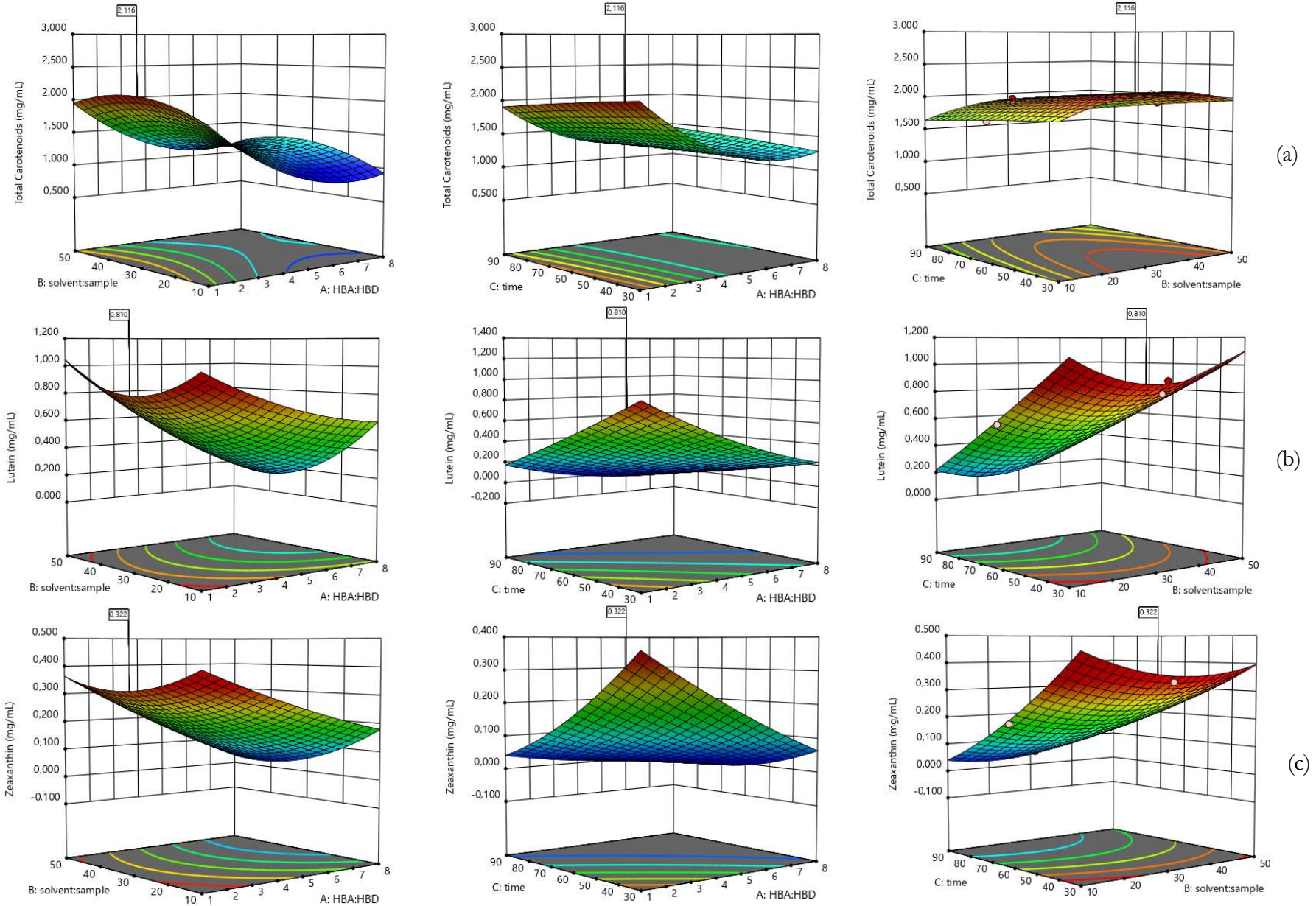
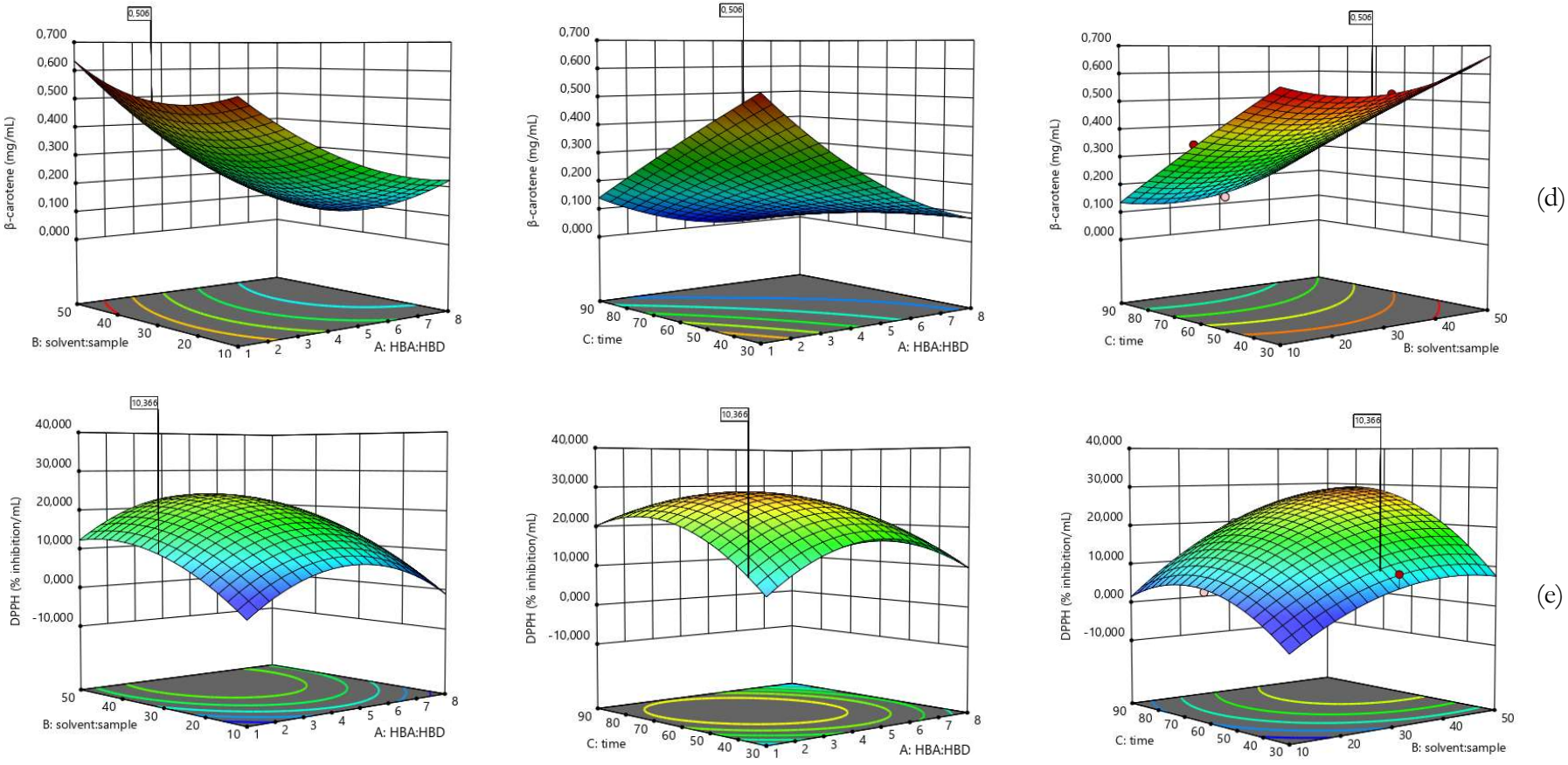


Figure 3.3. Surface Plots of the fitted polynomial equations (Eq. 3.4-3.7) for total carotenoids, lutein, zeaxanthin, β-carotene, and DPPH.



(d)

(e)

Figure 3.3. **(continued)** Surface Plots of the fitted polynomial equations (Eq. 3.4-3.7) for total carotenoids, lutein, zeaxanthin, β-carotene, and DPPH responses.

3.3.3.3. Validation of the predicted model

Starting from the data obtained for each response, the extraction process was optimized with reference to the level of the investigated variables using a specific software. In order to force the selection of parameters mainly related to the economic sustainability of the process, a constraint function was applied to the variables solvent:sample ratio and extraction time, as long as the desirability function of the individuated solution remained over 0.70. On these basis, the resultant optimal values for HBA:HBD molar ratio, solvent:sample ratio, and extraction time were 1:1, 27.33:1, and 33 minutes, respectively, with a desirability value equal to 0.725. Validation of the model was obtained by carrying out an extraction test using the above-mentioned conditions, and the results are reported in Table 3.5, along with the values predicted by each specific equation and the percentage of Fit (% Fit). This latter parameter was found to be very high for all the investigated responses (> 72 %), confirming how the selected model adequately fitted the experimental data.

Table 3.5. Predicted and actual experimental values of the investigated responses under the optimal extraction conditions for each extraction process.

	Predicted Value (mg/mL)	Experimental Value* (mg/mL)	% Fit
Total Carotenoids	2.116	1.996 ± 0.306	94.329 %
Lutein	0.910	1.421 ± 0.005	156.154 %
Zeaxanthin	0.222	0.160 ± 0.001	72.072 %
β-carotene	0.506	0.699 ± 0.002	138. 142 %
DPPH	10.366	9.133 ± 0.177	88.105 %

* Results are expressed as the mean value of three independent experiments ± standard deviation.

3.4. Conclusions

Seven low viscous NaHDESs, exclusively made of fatty acids that can act as hydrogen bond donors and acceptors simultaneously, were physicochemical characterized and tested for the extraction of carotenoids from the microalga *Chlorella vulgaris*. The screening step allowed the selection of nonanoic/dodecanoic acid (3:1) NaHDES as the potentially best extracting media, and its HBA:HBD molar ratio was optimized by implementing the BBD and RSM. The other two extraction parameters taken into account in the optimization step were solvent:sample ratio and extraction time, and the effect of these variables was evaluated considering the amounts recovered of carotenoid, with special regard to lutein, zeaxanthin, and β-carotene, as well as the antioxidant activity. Working at the optimized extraction conditions, the recovered amounts of total carotenoids, lutein, zeaxanthin, and β-carotene were 1.996 ± 0.306, 1.421 ± 0.001, 0.160 ± 0.001, 0.699 ± 0.002 mg/mL of extract, respectively. The extract showed

also an interesting level of antioxidant capability, assessed in 9.133 ± 0.177 % of inhibition/mL of extract. These results indicated an overall better performance of the NaHDES solvent in comparison with acetone, and confirm the potentialities of NaHDESs as a promising alternative to traditional organic solvents for the recovery of valuable bioactive compounds.

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3.6. Supplementary

Table S3.1. Experimental densities of the investigated NaHDESs as function of temperature.

	Density (ϱ, g·cm^3)*						
	20 °C	30 °C	40 °C	50 °C	60 °C	70 °C	80 °C
HDES 12	0.900	0.895	0.891	0.880	0.876	0.871	0.866
HDES 13	0.902	0.898	0.892	0.884	0.873	0.867	0.862
HDES 14	0.863	0.861	0.853	0.846	0.840	0.836	0.831
HDES 15	0.902	0.900	0.892	0.885	0.878	0.874	0.868
HDES 16	0.858	0.856	0.849	0.842	0.836	0.831	0.827
HDES 17	0.892	0.889	0.883	0.875	0.869	0.864	0.859
HDES 18	0.896	0.893	0.886	0.880	0.873	0.866	0.861

* Results are expressed as the mean value of two independent measurements.

Table S3.2. Parameters, a and b of Equation (3.2) and respective correlation coefficient (R^2), describing temperature dependence of density of the investigated NaHDESs.

	a (g·cm^3)	b (g·cm^3·K)	R^2
HDES 12	1.0744	-0.0006	0.9853
HDES 13	1.1144	-0.0007	0.9827
HDES 14	1.0325	-0.0006	0.9866
HDES 15	1.0776	-0.0006	0.9880
HDES 16	1.0227	-0.0006	0.9906
HDES 17	1.0637	-0.0006	0.9915
HDES 18	1.0765	-0.0006	0.9934

Table S3.3. Experimental viscosities of the investigated NaHDESs as function of temperature.

	Viscosity (mPa·s)*								
	20 °C	25 °C	30 °C	35 °C	40 °C	45 °C	50 °C	55 °C	60 °C
HDES 12	9.841	8.089	7.342	6.865	6.007	5.396	5.196	5.198	5.019
HDES 13	9.642	7.873	6.183	5.493	4.803	4.353	3.934	3.534	3.334
HDES 14	8.786	7.587	6.699	5.700	5.051	4.523	4.135	3.999	3.927
HDES 15	12.591	11.592	10.704	9.705	8.616	7.728	7.440	7.260	7.232
HDES 16	15.562	14.573	13.484	12.495	11.816	11.127	10.138	9.849	9.660
HDES 17	17.121	16.132	15.253	14.154	13.265	12.576	12.087	11.798	11.687
HDES 18	13.583	12.594	12.383	11.693	11.080	10.527	9.867	9.592	9.480

Table S3.4. Parameters, A_η B_η, and C_η of Equation (2.3) and respective correlation coefficient (R^2), describing temperature dependence of viscosity of the investigated NaHDESs.

	Aη (mPa·s)	Bη (mPa·s)	Cη (K)	R^2
HDES 12	0.4573	16.531	262.200	0.9885
HDES 13	0.0158	42.403	249.444	0.9977
HDES 14	0.0040	57.616	232.628	0.9981
HDES 15	0.2793	67.814	211.174	0.9807
HDES 16	0.2604	121.429	163.185	0.9947
HDES 17	0.7435	34.433	223.440	0.9908
HDES 18	0.0606	237.768	71.174	0.9879

4. Physicochemical and rheological characterization of cocoa and hazelnut spreadable creams fortified with carotenoid-rich NaHDES extracts

The content of this chapter was published in:

27th Workshop on *the Developments in the Italian PhD Research on Food Science Technology and Biotechnology,* University of Naples, Portici (2023). **Sportiello, L.** Optimization of the extraction techniques using Natural Hydrophobic Deep Eutectic Solvents for the recovery of biomolecules from food and food industry by-products. Oral Presentation.

4.1. Introduction

According to their definition, discretionary food products are not essential for human health. They are generally characterized by high energy levels, mainly associated with fats and sugars, and scarcity of nutrients such as vitamins and fibers. For this reason, the overconsumption of these food items is linked to an increased risk of obesity and chronic diseases, including heart disease, stroke, type 2 diabetes, and cancer (Sui et al., 2017; Livingstone et al., 2021). Despite the relevance of reducing the intake of these energy-dense, nutrient-poor foodstuffs and drinks, there is no general agreement on their definition and classification (Biltoft-Jensen et al., 2022). However, foods with high fat and sugar content, such as candy, biscuits, desserts, ice cream, and spreadable sweet creams, are generally recognized as belonging to this category. Among the several strategies that have been developed to reduce the negative impact of these products, fortification can represent a valuable approach to mitigating the undesired characteristics and improving the nutritional value without compromising their textural and sensory attributes.

A few papers have studied spreadable sweet creams' fortification with minerals, vitamins, aminoacids, and proteins. No-added sugars and reduced-fat spreadable cream products were investigated as a substrate, also using microencapsulation as a potential fortification technique (Yeh et al., 2002; Stathopoulos et al., 2009; Tolve et al., 2021; Tolve et al., 2022). Additionally, in the study of Cascone et al. (2023), chestnut sweet cream was fortified with carotenoids and inulin, improving the nutritional profile of the base chestnut cream product in terms of dietary fibers, carotenoid content, and antioxidant activity. However, none of the reported works have explored the use *'as such'* of NaHDEs-based extracts rich in bioactive compounds. At present, only one study has investigated the addition of natural hydrophilic DES extracts rich in polyphenols to fortify milk-based chocolate drinks (Panić et al., 2020).

This novel approach for fortification in the food industry can be a promising technique, taking advantage of the food-grade nature of NaDES and eliminating the need to remove the extraction solvent. Therefore, in this study, carotenoids derived from carrot peels and *Chlorella vulgaris* microalga were extracted using two NaHDESs and used as functional ingredients for fortifying a commercial hazelnut and cocoa spreadable cream.

Initially, the NaHDES-based extracts were characterized by studying the antioxidant stability during storage at different environmental conditions in order to evaluate the preservation effect of these solvents. The results were also compared with those of extracts obtained at the same extracting operating conditions but using acetone as a solvent. Then, four formulations of fortified creams were obtained by adding two different amounts of each extract to the hazelnut and cocoa spreadable cream. The newly formulated creams were characterized for oil separation, a_w, color, rheological and textural properties, carotenoid content, and antioxidant activity. The data were compared to the control formulation to assess the effects of the fortification.

4.2. Material and Methods

4.2.1. Standards, reagents and solvents

Dodecanoic acid (≥ 98.0 %), nonanoic acid (≥ 96.0 %), DL-menthol (≥ 98.0 %), and thymol (≥ 98.5 %) were used for the NaHDESs preparation. Furthermore, control samples for the assessment of the antioxidant stability were obtained using acetone (≥ 99.8 %) as a solvent The 2,2-diphenyl-1-picrylhydrazyl, 2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS ≥ 98 %), potassium persulphate (98 %), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox 97 %) were used for the antioxidant assays. Acetonitrile (≥ 99.9 %), triethylamine (≥ 99.8 %), and β-carotene (96.4 %), lutein (95.7 %) and zeaxanthin (97.5 %) standards were used for the HPLC analysis. All the chemicals were purchased from Merck KGaA (Darmstadt, Germany). Ultrapure water was produced using a Milli-Q system (Millipore, Billerica, MA, USA).

4.2.2. NaHDESs preparation and characterization

The NaHDESs thymol/DL menthol (1:4) and nonanoic acid/dodecanoic acid (1:1) were prepared following the method proposed by Dai et al. (2013), with slight modifications. The HBA and the HBD were combined in the above-mentioned specific molar ratios and mixed at 750 rpm under mild heating (60 °C) until a clear transparent liquid was obtained. Afterward, the resulting solvents were gradually

cooled to room temperature and then characterized to assess their density, dynamic viscosity, and rheological behavior.

4.2.2.1. Density

NaHDESs density was measured utilizing a pycnometer (Merck KGaA, Darmstadt, Germany).

4.2.2.2. Viscosity and rheological behavior

The rheological characteristics of the prepared NaHDESs were evaluated using a DSR 500 CP4000 rheometer (Lamy Rheology, Champagne-au-Mont-d'Or, France). In all cases, viscosity measurements were carried out using the measuring system MS-DIN11, applying different shear-rates, ranging from 50 to 300 s^{-1}. The viscosity values were fitted to the Power Law model as a function of shear-rate (Eq. 4.1) to calculate the flow behavior (n) and flow consistency (K) indices (Macosko, 1994).

$$\eta(\dot{\gamma}) = K \cdot \dot{\gamma}^{n-1} \qquad (Eq. 4.1)$$

where η is the viscosity (mPa·s), K is the flow consistency index (mPa·s) representing the viscosity at the shear-rate $\dot{\gamma} = 1\ s^{-1}$, and n is the power law index (adimensional) defining the steepness of the shear thinning decay for $n < 1$ (Eberhard et al. 2019).

4.2.3. Extracts preparation and characterization

The NaHDES-based extracts rich in carotenoid were obtained carrying out the extraction from two different matrices, namely the peels of fresh carrots and the biomass of *Chlorella vulgaris*. The ultrasound-assisted extractions, whose optimal parameters were identified in previous studies (see Sections 2.3.6 and 3.3.6), were conducted using thymol/DL-menthol (1:4) for the extraction from carrot peels (solvent:sample 10:1) and nonanoic/dodecanoic acid (1:1) for the extraction from *Chlorella vulgaris* (solvent:sample 27.33:1). After the addition of the lyophilized samples to the solvents, the mixtures were vortexed at 25 °C for 60 seconds and then sonicated at 45 kHz and 50 °C for 30 minutes in the case of carrot peels, and 33 minutes for *Chlorella vulgaris*, using a 2200 MH S3 sonication unit (SOLTEC, Milan, Italy). The extracts were then centrifuged at 3900 RCF for 10 minutes. All manipulations were carried out shading the samples, to minimize carotenoid photodecomposition throughout the analytical procedure. The extracts were then stored at -20 °C until further analyses. Acetone extractions were also carried out under the same experimental conditions as a reference. All extractions were performed in triplicate, and the results were expressed as the mean value ± standard deviation.

Afterward, the obtained carotenoid-rich NaHDES extracts were characterized for their carotenoid content, carrying out UV-Vis spectrophotometric measurements and HPLC analysis to assess lutein, zeaxanthin, and β-carotene yields, as well as their antioxidant activity, using ABTS and DPPH assays.

4.2.3.1. Carotenoid and antioxidant activity determination

4.2.3.1.1. UV-Vis spectrophotometry

The total carotenoid content in the extracts was assessed spectrophotometrically after diluting an aliquot in acetone (1:50 v/v). The spectra were acquired over the 350-750 nm range, using a V-750 UV-Visible Spectrophotometer (Jasco, Oregon, USA), and the obtained data were then fitted using the 'Solver' add-in of Excel, according to the method proposed by Chazaux et al. (2022). The total carotenoid content was calculated and expressed as mg/mL of extract..

4.2.3.1.2. HPLC-DAD analysis

Carotenoid analysis was carried out according to the method described by Perozeni et al. (2020). Lutein, zeaxanthin, and β-carotene were tentatively identified and quantified using a C18 column (Gemini 3 µm C18 110 A 50x4,6 mm, Phenomenex) and an LC-4000 system with an MD-4010 PDA detector (Jasco, Oregon, USA). Moiety separation was achieved by gradient elution [A to B from 0 to 100% in 15 min, where A= ethyl acetate, B= acetonitrile–water–triethylamine (9:1:0.01 v/v/v)] at the flow rate of 1.5 mL/min and recording the chromatogram at of 440 nm. Lutein, zeaxanthin, and β-carotene identification was achieved by comparing their retention times and spectra to those obtained using commercially available standards (Extrasynthese, Genay, France). Furthermore, pigment quantification was carried out using the external standard technique.

4.2.3.1.3. Antioxidant Activity

The antioxidant activity of the extracts was assessed using both the 2,2'-azinobis-(3-ethylenebenzothiazoline)-6-sulfonic acid (ABTS) assay and the 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH) assay. ABTS determination was performed according to the method of Re et al. (1999), with slight modifications. After preparation, the $ABTS^{\bullet+}$ stock solution was diluted with H_2O, pH 7.4, to an absorbance of 0.70 (± 0.02) at 734 nm. Subsequently, 9.8 mL of $ABTS^{\bullet+}$ solution were mixed with 200 µL of sample in a 15 mL tube and stirred for 60 minutes at room temperature. The spectrophotometric measurement was performed at 734 nm, and the antioxidant capacity was expressed in Trolox equivalent per milliliter of extract (mM TE/mL of extract).

The DPPH assay was carried out following the method proposed by Maggini et al. (2018). Thirty μL of extracts were placed in tubes with 2.97 mL of DPPH solution (20 mg/L DPPH in MeOH). After 45 minutes of incubation at room temperature, in dark conditions, and under continuous mixing, the antioxidant power was measured spectrophotometrically at 515 nm. The percentage inhibition of the DPPH radical per milliliter of extract was calculated from the absorbance values of the blank (A_{blank}) and of the sample (A_{sample}) as follows:

$$\%\ \text{Inhibition}\ \text{mL}^{-1} = 100 \cdot [(A_{blank} - A_{sample}) / A_{blank})] / \text{mL}$$

4.2.3.1.4. Carotenoid and antioxidant stability

For the stability test, the effects of storage time, light exposure, and temperature on total carotenoids and antioxidant capacity of the carotenoid-rich NaHDES extracts were monitored, with acetonic extracts used as controls. The extracts were stored under light (at 25 °C) and in the dark (at 25, 5, and −20 °C), obtaining for different conditions. The extracts' total carotenoid and antioxidant capacity were assessed every two weeks over 45 days using the methods described in Sections 4.2.3.1.1 and 4.2.3.1.3. The results were expressed according to the degradation rate (C/C_0), where C_0 is the initial carotenoid concentration and antioxidant capacity, and C is the carotenoid concentration and antioxidant capacity after storage. All experiments were performed in triplicate.

4.2.4. Cocoa and hazelnut spreadable cream fortification and characterization

Cocoa and hazelnut spreadable cream "Nocciocrem" by Gandola (Brescia, Italia) was purchased in a local market. The product was heated to 60 °C for 5 minutes to prepare the fortified creams and then cooled to 45 °C. By keeping the temperature constant, the extracts were incorporated under continuous stirring until a perfectly blended mass was obtained. Afterward, the samples were cooled to room temperature and stored in the dark until analyzed.

Two fortified cream formulations were produced, adding the two different carotenoid-rich NaHDES extracts, while the base cream without the extract was used as the control formulation. The acronyms, descriptions, and concentrations of the formulations are reported in Table 4.1. All formulations were prepared in duplicate.

4.2.4.1. Physicochemical and rheological analysis

The obtained formulations were characterized by evaluating the accelerated oil separation, water activity, color, textural properties, and rheological measurements.

4.2.4.1.1. Accelerated oil separation, water activity, and color

The procedure used to perform the accelerated oil separation (AOS) was adapted from Aryana et al. (2000). An aliquot (7.5 g) of the sample was weighed into a test tube and centrifuged (10 minutes at 4600 RCF). The separated oil was recovered and weighed. The AOS was expressed as g oil/100 g cream.

The cocoa and hazelnut creams' water activity (a_w) was assessed using a HygroPalm HP23-AW-A device equipped with a HC2-AW measurement station (Rotronic, Bassersdorf, CH). Lastly, for color measurements, the parameters L*, a*, and b* were acquired using a handheld Minolta Chroma meter (CR-3000, Konica Minolta, Japan).

Table 4.1. Cream formulations with acronyms, descriptions, and concentrations.

Acronym	Description	NaHDES extract (mL/100g)
CC	cream base	-
CA	cream base + carrot peels NaHDES (thymol/DL menthol) extract	28
CB	cream base + carrot peels NaHDES (thymol/DL menthol) extract	12
CD	cream base + *Chlorella vulgaris* NaHDES (nonanoic acid/dodecanoic acid) extract	28
CE	cream base + *Chlorella vulgaris* NaHDES (nonanoic acid/dodecanoic acid) extract	12

4.2.4.1.2. Rheological measurements

The rheological behavior of cocoa and hazelnut cream samples was measured with a rheometer (DSR 500 CP4000 Rheometer, Lamy Rheology, Champagne-au-Mont-d'Or, France) using concentric cylinders (MS-DIN11) as recommended by the International Confectionery Association, ICA (2000). The measurement of stress and viscosity were made at shear rates between 2 s^{-1} and 50 s^{-1} with a ramp up and down in shear rate. All the measurements were carried out at 40 °C. Each sample was analyzed in triplicate. The viscosity was calculated by interpolation of the data obtained using the Rheotex software (Lamy Rheology Champagne-au-Mont-d'Or, France), implementing the Casson regression model

[Equation (4.1)]. Power-law model was also used to describe the flow behavior of different formulations according to Equation (4.2).

$$\tau^{0.5} = \left(\tau_y\right)^{0.5} + \eta_{pl}(\dot{\gamma})^{0.5} \qquad Eq\ (4.1)$$

$$\tau = \mathrm{K} \cdot \dot{\gamma}^{n} \qquad Eq\ (4.2)$$

where τ is the shear stress (Pa), τ_y is Casson's yield stress (Pa), η_{pl} is Casson plastic viscosity (mPa•s), K is the consistency coefficient (mPa*s), $\dot{\gamma}$ is the shear rate (s^{-1}) and n is the flow behavior index (dimensionless).

4.2.4.1.3. Textural properties

Spreadability and stickiness analyses were performed by using a TX-700 Texture Analyzer (Lamy Rheology, Champagne-au-Mont-d'Or, France). Cocoa and hazelnut cream samples were analyzed at room temperature (25 ± 0.5 °C), using a 5N probe with a pre–sets distance of 25 mm, test speed 1 mm/s, compression distance 15 mm. The data obtained were calculated using the Rheotex software (Version 2023, Lamy Rheology).

4.2.4.2. Carotenoid and antioxidant activity extraction and determination

Total carotenoids and the antioxidant activity using ABTS and DPPH of cocoa and hazelnut cream samples were evaluated using the procedures described in Sections 4.2.3.1.1 and 4.2.3.1.2, after pre-treating the samples to extract the carotenoids fraction. The extraction was carried out by adapting the procedure described by Cascone et al. (2023). 12.5 mL of hexane/ethanol/acetone (2:1:1, v/v) solution was added to 5 g of cream. Afterward, the mixture was sonicated for 10 minutes, then centrifuged for 10 minutes at 2500 RFC, and the supernatant recovered. The procedure was repeated three times. The supernatants were pooled and transferred to a 250 mL separatory funnel and, in order to avoid emulsion formation, the acetone was removed by adding 5 mL of ultrapure water (Milli-QMillipore), and the upper solution was recovered for carotenoids analysis.

4.2.4. Statistical analysis

Statistical analysis of the data was performed using the software XLSTAT Premium (Version 2020.3.1, Addinsoft, Paris, France) applying one-way ANOVA. Significant differences between means were computed by Tukey's HSD (Honestly Significantly Different) test at 95% confidence interval. In addition, the fitting of the equation 4.1 and 4.2 were obtained using the software Rheotex (Version 2023, Lamy

Rheology, Champagne-au-Mont-d'Or, France), while the plots were obtained using OriginPro software (Version 2023, OriginLab Corporation, Northampton, Ma, USA).

4.3. Results and discussion

4.3.1. NaHDESs preparation and characterization

In the present study, two NaHDESs were obtained by combining food-grade starting materials at specific molar ratios. HBAs/HBDs, molar ratios, and physicochemical characterization are reported in Table 4.2. The obtained density, viscosity, and rheological behavior data were found to be in agreement with those reported in the literature (Florindo et al., 2018; Kyriakoudi et al., 2022; Sportiello et al., 2023) and the previous experimental results (see Sections 2.3.1 and 3.3.1). As expected, the thymol/DL-menthol NaHDES density and viscosity at 25 °C (0.913 ± 0.006 and 64.283 ± 0.244, respectively) were higher than those assessed for the nonanoic acid/dodecanoic acid NaHDES (0.892 ± 0.005 and 13.165 ± 0.455, respectively). Furthermore, by assessing NaHDESs viscosity in the shear-rate range 50 – 300 s^{-1}, a shear-thinning behavior was identified in the imposed shear-rate range, as shown by the "n" values calculated according to the Equation (4.1) (see Section 4.2.2.2), and reported in Table 4.2.

Table 4.2. HBAs/HBDs, molar ratios, and physicochemical characterization of the prepared NaHDESs.

HBA/HBD	Molar Ratio	Density* (g/cm^3 at 25 °C)	Viscosity* (mPa·s at 25 °C)	Flow Behavior Index (n) *	Flow Consistency Index (K) *
thymol/DL-menthol	1:4	0.913 ± 0.008	64.283 ± 0.244	0.940	78.795
nonanoic acid/dodecanoic acid	1:1	0.892 ± 0.005	13.165 ± 0.455	0.761	32.389

* Results are expressed as the mean and standard deviation values of three independent measurements.

4.3.2. Extracts characterization

Taking into account the safety of the NaHDES-based extract as a prerequisite for their use in food applications, a characterization of their carotenoid content and their antioxidant stability during storage was carried out before evaluating the fortification. Specifically, total carotenoids, lutein, zeaxanthin, β-carotene, and the antioxidant activity of the obtained carotenoid-rich NaHDES extracts were assessed and the results are reported in Table 4.3, together with the data obtained for acetonic extracts used as reference. Compared with the extractions carried out with acetone, the results showed that the thymol/DL-menthol NaHDES could extract 86.94 and 82.48% of the available carotenoids and β-carotene, respectively. Instead, the nonanoic acid/dodecanoic acid NaHDES showed better

performances, allowing recoveries of 119.38, 132.93, 91.43, and 163.70% for total carotenoids, lutein, zeaxanthin, and β-carotene, respectively. Regarding the ABTS and DPPH values, the data indicate a higher antioxidant activity for the NaHDES extracts compared to those obtained when using acetone as the solvent. These results agree with those obtained in the previous steps of this research (see Chapters 2 and 3).

Table 4.3. Characterization of the carotenoid-rich NaHDESs extracts and acetonic extracts.

Extract	Total Carotenoids (mg/mL)	Lutein (mg/mL)	Zeaxanthin (mg/mL)	β-Carotene (mg/mL)	ABTS (mmol TE /mL)	DPPH (% inhibition/mL)
thymol/DL-menthol + carrot peels	3.534 ± 0.147	-	-	3.296 ± 0.126	650.154 ± 0.121	43.374 ± 1.236
nonanoic acid/dodecanoic acid + *Chlorella vulgaris*	1.996 ± 0.306	1.421 ± 0.005	0.160 ± 0.001	0.699 ± 0.002	300.154 ± 0.121	9.102 ± 1.236
acetone + carrot peels	4.065 ± 0.028	-	-	3.996 ± 0.026	578.226 ± 0.167	45.989 ± 0.563
acetone + *Chlorella vulgaris*	1.672 ± 0.299	1.069 ± 0.134	0.175 ± 0.018	0.427 ± 0.053	270.154 ± 0.133	7.612 ± 1.236

4.3.2.1. Carotenoid and antioxidant stability

The carotenoid and antioxidant stability of the NaHDES-based extracts was investigated, since the role of DESs in improving the stability of the target bioactive compounds during storage was documented by previous research (Stupar et al., 2021; Vinas-Ospino et al., 2023; Vinas-Ospino et al., 2023). This behavior is due to the interaction between the solvent and the target compounds, mainly represented by hydrogen bonding, which limits the flow of solute molecules and minimizes oxidative degradation. In addition, the contact of the solute molecules with oxygen at the interface between the DESs and air is minimized (Cvjetko Bubalo et al., 2018). Based on these evidences, the carotenoid and antioxidant stability were monitored for 45 days under different conditions and temperatures, using the acetonic extracts as controls. The results of this investigation are presented in Figures 4.1, 4.2, and 4.3. As can be observed, regardless of the solvent used for the extraction, the results obtained for total carotenoids, ABTS and DPPH converged in indicating a faster degradation of the extracts exposed to light than those stored in the dark. Furthermore, considering the storage in dark conditions at the investigated temperatures, ANOVA analysis was implemented to evaluate if low temperatures are not needed to preserve the carotenoids and the antioxidant stability. The obtained results showed divergent responses since the antioxidant assays didn't show significant differences in most cases, while the carotenoid content was significantly affected by temperature in all cases, with the lower degradation occurring at the lowest temperature. In addition, aiming to highlight the possible preservation effect of NaHDESs, in each condition, the comparison between the NaHDES extracts and the equivalent acetone extracts was carried out obtaining the following results:

- Under light conditions at 25 °C, thymol/DL menthol NaHDES played a significant preservative role, maintaining approximately 50-80 % of the initial carotenoids and antioxidant activity after 45 days. In contrast, the equivalent extract obtained using acetone only retained about 10 % of these properties. Differently, no preservation effect was observed for nonanoic acid/dodecanoic acid, except for a slight minor degradation of total carotenoids as compared to the acetone extractions;
- The same general trends were appreciated under dark conditions at 25, 4, and -20 °C, exerting only the thymol/DL-menthol NaHDES a preservative effect. More specifically, better results were obtained when the extracts were kept at 4 and -20 °C, with no significant differences in the degradation of carotenoids and antioxidant stability.

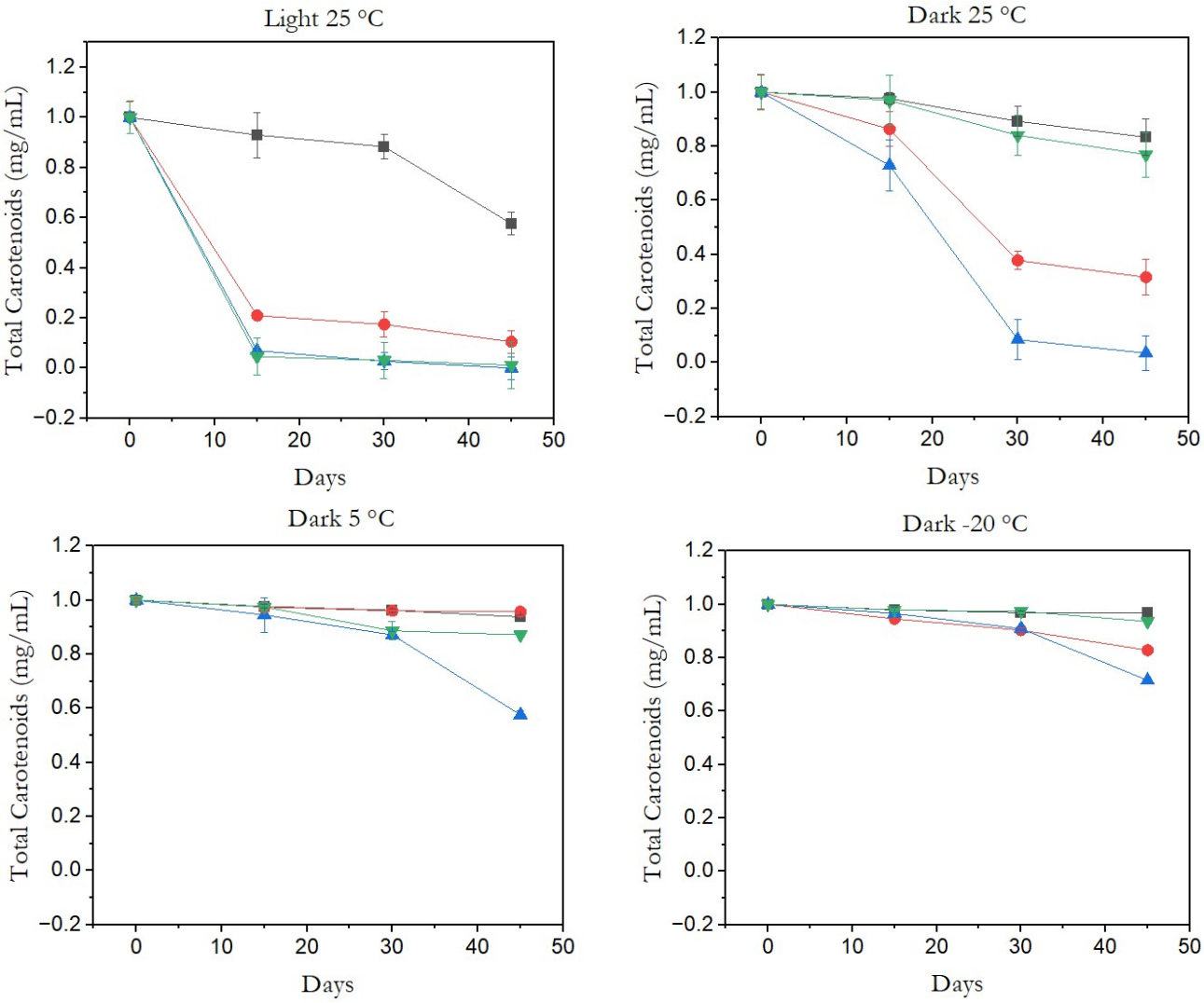

Figure 4.1. Total carotenoids stability of thymol/DL-menthol-carrot peels extract (black), nonanoic acid/dodecanoic acid-*Chlorella vulgaris* extract (red), acetone-carrot peels extract (blue), and acetone-*Chlorella vulgaris* extract (green) under light at 25 °C and in dark condition at 25, 5, and -20 °C.

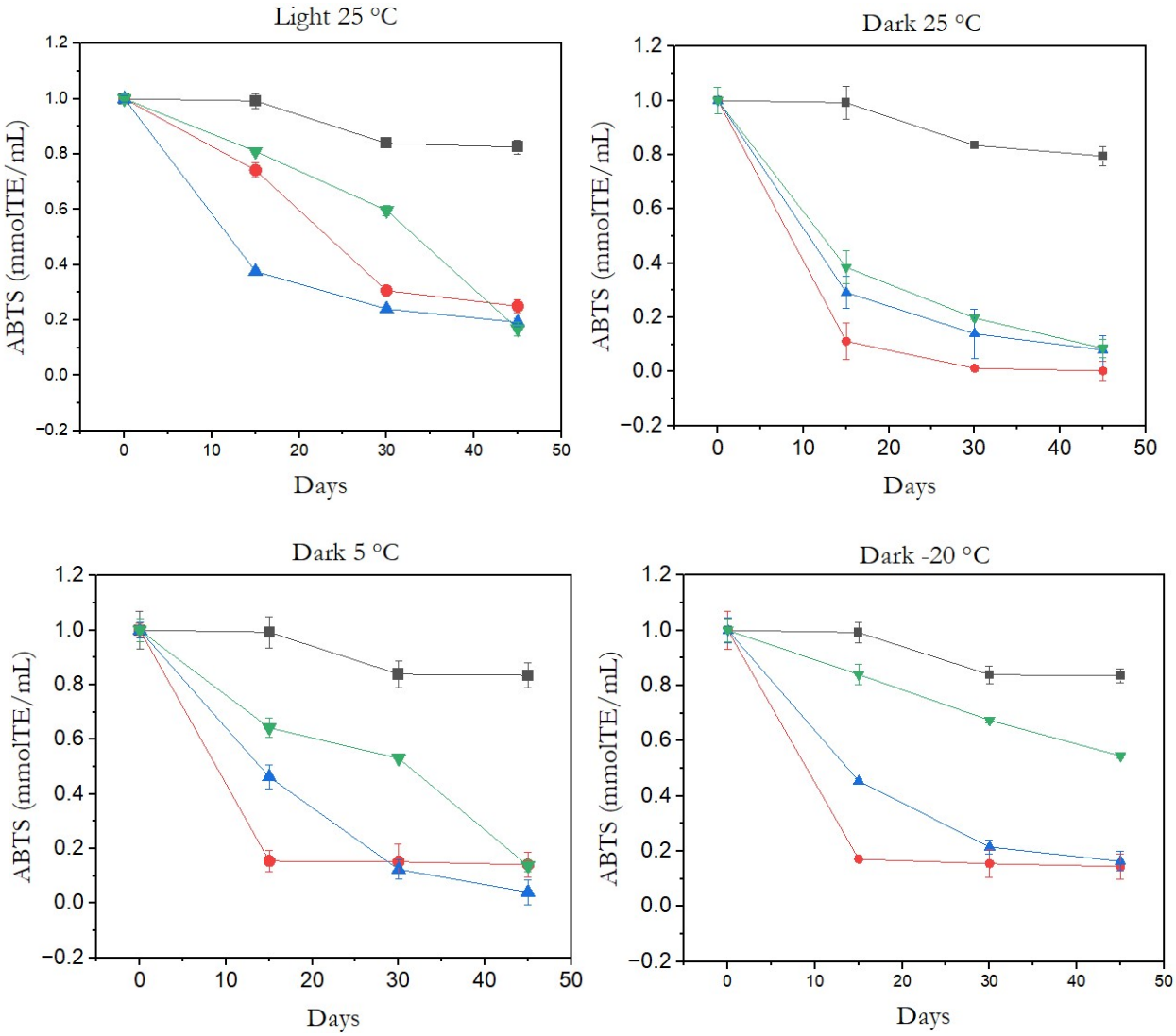

Figure 4.2. Antioxidant stability assessed with ABTS assay of thymol/DL-menthol-carrot peels extract (black), nonanoic acid/dodecanoic acid-*Chlorella vulgaris* extract (red), acetone-carrot peels extract (blue), and acetone-*Chlorella vulgaris* extract (green) under light at 25 °C and in dark condition at 25, 5 and -20 °C.

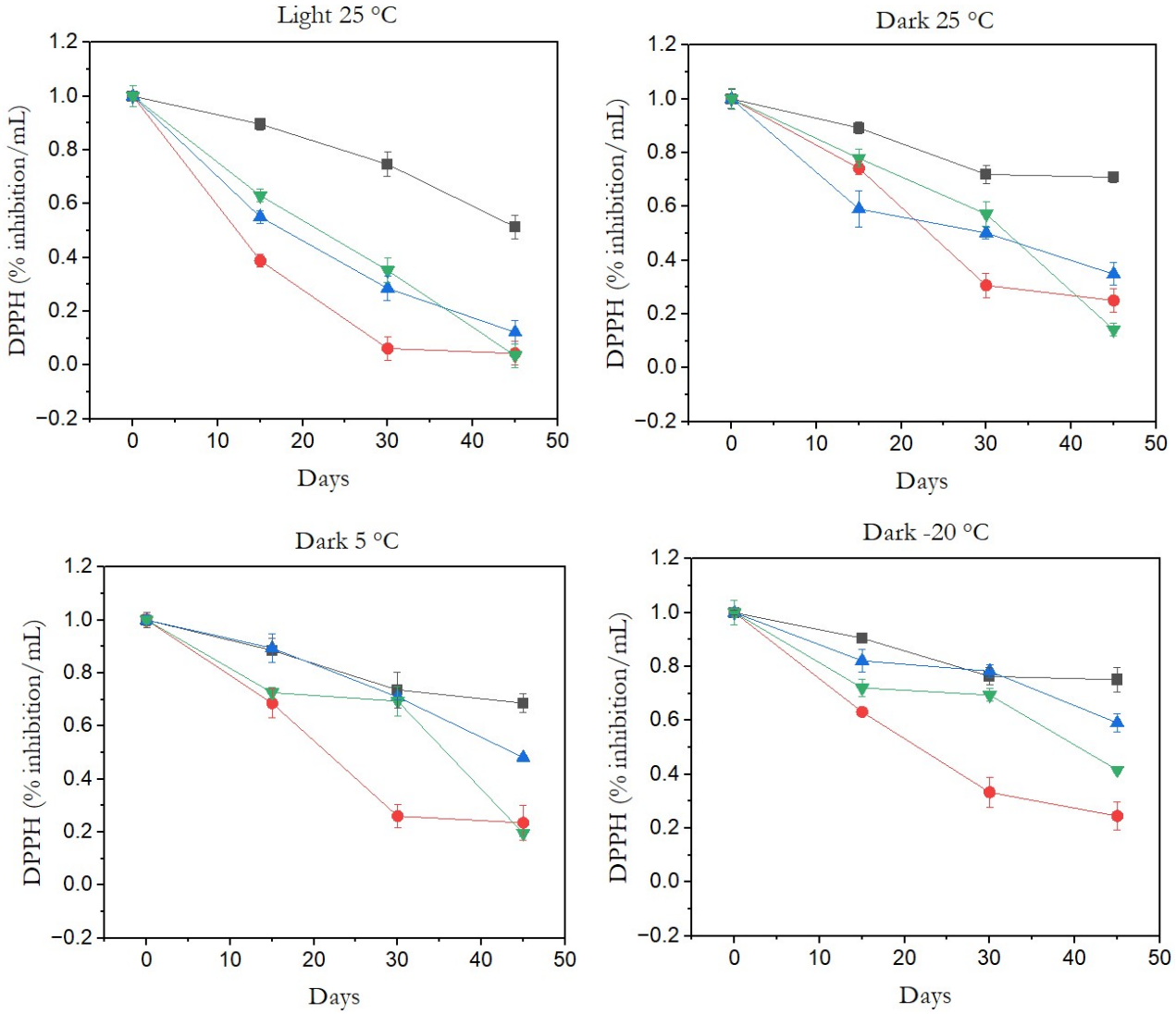

Figure 4.3. Antioxidant stability assessed with DPPH assay of thymol/DL-menthol-carrot peels extract (black), nonanoic acid/dodecanoic acid-*Chlorella vulgaris* extract (red), acetone-carrot peels extract (blue), and acetone-*Chlorella vulgaris* extract (green) under light at 25 °C and in dark condition at 25, 5 and -20 °C.

Based on these results, it can be concluded that the thymol/DL-menthol NaHDES can play an interesting preservation role with respect to a more traditional solvent such as acetone. This protective action was enhanced at low temperatures and in the absence of light. After 45 days of storage in the dark at -20 °C, the extract preserved 96.70, 85.52, and 75.11% of the initial total carotenoids, ABTS, and DPPH values, respectively, against 71.53, 16.36, and 59.05% retained in the acetonic extract.

4.3.3. Fortified cocoa and hazelnut spreadable cream characterization

Using the base cream as control, the fortified spreadable creams were characterized for oil separation, water activity, color, textural properties, and rheological properties. The results are reported in Tables 4.4 and 4.5.

4.3.3.1. Accelerated Oil Separation, Water Activity, and Color

AOS, water activity, color, and TSS of newly formulated cocoa and hazelnut spreadable creams with carrot peels NaHDES (thymol/DL menthol 1:4) extracts (CA and CB) are presented in Table 4.4, while the same parameters of other two fortified creams with *Chlorella vulgaris* NaHDES (nonanoic acid/dodecanoic acid 1:1) extracts (CD and CE) are presented in Table 4.5. For comparison, the data obtained for the base cream (CC) are reported in both tables. Considering the formulations CA and CB, AOS values were statistically different between them and with respect to the control CC, ranging from 6.51 to 18.57 g/100 g. The lowest AOS value was found in sample CC (6.51 %), while an increase in the oil separation directly related to the amount of extract added was observed for the fortified samples CA (28 %) and CB (12 %). for which the recorded AOS values were 18.04 and 13.13 %, respectively.

This phenomenon could be ascribed to the fact that the base cream contains, besides other ingredients, about 25 % sunflower oil and lecithin as emulsion stabilizer. The added NaHDES solution might negatively interact with the lecithin, reducing its emulsion properties and releasing some oil. Furthermore, the heating and cooling steps utilized to prepare the samples for fortification, could have affected the cream physical stability inducing deoiling.

While the chemical nature of the added extracts can induce oil exudation, no effect was expected concerning the water activity value, which represents a crucial parameter for the microbiology and physical stability of the product (Roudaut, G. 2020; Tapia et al., 2020). The data obtained for CA and CB samples resulted in having very similar values with reference to the CC sample, with no statistical differences ($p > 0.05$), while in CD and CE a narrow decrease was observed, statistically significant only for CD sample.

Lastly, considering the L* a*, and b* color parameters, significant differences have been highlighted despite the strong masking effect of the brown color of the base cream. Specifically, CA, CB, CD, and CE samples were characterized by increased brightness (L) and an increase in the a values to yellow/orange color, especially for samples CA and CB. Additionally, in CD and CE b values were lower than the control, indicating an increase in green color. These results are consistent with the colors of the extracts, with the carrot peel extract being orange and the *Chlorella vulgaris* extract being green.

Table 4.4. Oil separation, water activity, color, rheological parameters, and textural properties of the spreadable cream samples fortified with carrot extract.

Investigated properties		Creams		
		CC	CA	CB
Accelerated Oil Separation (AOS) (g/100g)		6.514 ± 0.136 [a]	18.57 ± 0.749 [b]	11.443 ± 0.532 [c]
Water activity (a_w)		0.395 ± 0.004 [a]	0.419 ± 0.037 [a]	0.399 ± 0.024 [a]
Color parameters	L*	16.186 ± 0.994 [a]	16.882 ± 0.200 [a]	16.695 ± 0.181 [a]
	a*	18.297 ± 0.085 [a]	18.048 ± 0.159 [a]	18.135 ± 0.304 [a]
	b*	18.801 ± 0.129 [a]	25.435 ± 0.849 [b]	22.721 ± 0.934 [b]
Rheological parameters	τ_y (Pa)	10.253 ± 0.173 [a]	0.282 ± 0.342 [b]	2.064 ± 0.877 [c]
	η_{pl} (Pa•s)	1.083 ± 0.125 [a]	0.586 ± 0.007 [b]	0.344 ± 0.027 [c]
	n	0.510 ± 0.014 [a]	0.535 ± 0.049 [a]	0.500 ± 0.042 [a]
	K (Pa•s^n)	13.85 ± 1.209 [a]	0.565 ± 0.346 [b]	2.575 ± 0.029 [c]
	Yield stress [1] (Pa)	26.155 ± 10.472 [a]	1.545 ± 1.025 [b]	9.745 ± 0.544 [c]
	High shear viscosity [2] (Pa•s)	3.965 ± 0.007 [a]	1.571 ± 0.075 [b]	2.430 ± 0.097 [c]
	Tixotropy [3] (Pa•s)	1.661 ± 0.240 [a]	0.251 ± 0.070 [b]	1.593 ± 0.035 [a]
Texture parameters	Spreadability (N/s)	2.397 ± 0.243 [a]	1.125 ± 0.446 [a]	1.923 ± 1.537 [a]
	Stickiness (N/s)	-4.088 ± 1.021 [a]	-0.795 ± 0.304 [b]	-2.018 ± 1.191 [ab]

Values with different letters within the same row are significantly different for $p < 0.05$. CC= base cream; CA= base cream + 28 % carrot peels NaHDES (thymol/DL menthol) extract; CB= base cream + 12 % carrot peels NaHDES (thymol/DL menthol) extract, τ_y= the Casson's yield stress; η_{pl}= Casson plastic viscosity; K= the flow consistency index; n= the flow behavior index. [1] Yield stress at a shear rate of 5 s^{-1}; [2] value of the viscosity at a shear rate of 40 s^{-1}; [3] difference between the viscosity measured at a shear rate of 40 s^{-1} during the ramp up and down in shear rate.

Table 4.5. Oil separation, water activity, color, rheological parameters, and textural properties of the spreadable cream samples fortified with *Chlorella vulgaris* extract.

Investigated properties		Creams		
		CC	CD	CE
Accelerated Oil Separation (AOS) (g/100g)		6.514 ± 0.136 [a]	18.036 ± 0.749 [b]	13.128 ± 0.532 [c]
Water activity (a_w)		0.395 ± 0.004 [a]	0.372 ± 0.005[b]	0.376 ± 0.006 [ab]
Color parameters	L*	15.767 ± 0.415 [a]	17.00 ± 0.042 [b]	16.78 ± 0.026 [c]
	a*	18.291 ± 0.141 [a]	13.382 ± 0.436 [b]	16.041 ± 0.035 [c]
	b*	18.812 ± 0.085 [a]	20.604 ± 0.410 [b]	20.225 ± 0.148 [b]
Rheological parameters	τ_y (Pa)	10.253 ± 0.125 [a]	1.201 ± 0.023 [b]	4.161± 0.065 [c]
	η_{pl} (Pa•s)	1.083 ± 0.125 [a]	0.146 ± 0.113 [b]	0.405 ± 0.187 [c]
	n	0.510 ± 0.014 [a]	0.520 ± 0.028 [a]	0.475 ± 0.007 [a]
	K (Pa•s^n)	13.85 ± 1.209 [a]	1.691 ± 0.240 [b]	5.982 ± 0.735 [c]
	Yield stress [1] (Pa)	26.155 ± 10.472 [a]	3.712 ± 0.269 [b]	12.042 ± 1.400 [c]
	High shear viscosity [2] (Pa•s)	3.965 ± 0.007 [a]	0.538 ± 0.041 [b]	1.867 ± 0.118 [c]
	Tixotropy [3](Pa•s)	1.661 ± 0.240 [a]	0.088 ± 0.094 [b]	0.431 ± 0.186 [c]
Texture parameters	Spreadability (N/s)	2.397 ± 0.243 [a]	1.055 ± 0.272 [b]	1.733 ± 0.095 [b]
	Stickiness (N/s)	-4.088 ± 1.021 [a]	-0.228 ± 0.301 [b]	-0.811 ± 0.443 [b]

Values with different letters within the same column are significantly different for $p < 0.05$. CC= base cream; CA= base cream + 28 % + *Chlorella vulgaris* NaHDES (nonanoic acid/dodecanoic acid) extract; CB= base cream + 12 % + *Chlorella vulgaris* NaHDES (nonanoic acid/dodecanoic acid) extract. τ_y= the Casson's yield stress; η_{pl}= Casson plastic viscosity; K= the flow consistency index; n= the flow behavior index. [1] Yield stress at a shear rate of 5 s^{-1}; [2] value of the viscosity at a shear rate of 40 s^{-1}; [3] difference between the viscosity measured at a shear rate of 40 s^{-1} during the ramp up and down in shear rate.

4.3.3.2. Rheological measurements and textural properties

Rheological measurements carried out by increasing the shear rate from 2 to 50 s^{-1} using concentric cylinders indicated that all samples exhibited a shear thinning behavior, which was consistent with previous reports (Fernandes et al., 2013; Aydemir & Atalar, 2019; Tolve et al., 2021). This assumption

was confirmed by the power-law model, which reveals a similar flow behavior index, n = 0.5 (Table 4.4 and 4.5). The flow of a material, calculated from Eq. (4.2), is generally described as shear thinning for n < 1, shear-thickening if n > 1, or Newtonian flow for n = 1. In this study, both Casson and power-law models were applied to the samples because the former is the recommended one by the International Office of Cocoa and Chocolate, while the latter provides further information regarding the flow and processability behavior of a product, given the correlation between the n value and Reynold number (Sivakumar, Prakash Bharti & Chhabra, 2006). As reported in Tables 4.4 and 4.5, the addition of carotenoid-rich NaHDES extracts caused a significant viscosity decrease with respect to the control formulation and also between the formulations for each extract. These results were expected considering the low viscosities values of the added extracts (see Section 4.3.1). The higher viscosity decrease observed for CD and CE samples than that shown by CA and CB can be explained by the lower viscosity of nonanoic acid/dodecanoic acid 1:4 NaHDES in comparison with thymol/DL-menthol 1:1 NaHDES. Considering the rheological parameters calculated by applying the Casson and power-law model, a relatively good correlation was found between the Casson plastic viscosity (η_y) and the suggested viscosity parameters. However, it is worth noting that the Casson's yield stress did not show the same level of correlation (Table 4.4 and 4.5). These findings are in line with the research conducted by Tolve et al. in 2021, which suggests that yield stress at 5 s^{-1} and viscosity at 40 s^{-1} of the shear rate are more reliable parameters to consider when studying the viscosity of cocoa-based products.

Lastly, observing the textural properties, the spreadability and stickiness values decreased as the percentage of the added extract increased ($p < 0.05$). The observed values ranged from 2.397 to 1.125 and -4.088 to -0.795 N/s for spredability and stickness, respectively in CC, CA and CB samples, while for CC, CD and CE the data varied from 2.397 to 1.055 and -4.088 to -0.228 N/s for spredability and stickness, respectively. This trend can be easily correlated to what was observed for the viscosity values. Samples CA and CD, with an amount of extract equal to 28 % (v/w), showed the lowest viscosity, corroborating the evidence that this percentage of NaHDES-based extracts strongly affected these properties.

4.3.3.3. Carotenoids and antioxidant activity

It is worth reporting that a given amount of carotenoids and some antioxidant activity characterized the base cream CC, in which sunflower oil, containing carotenoids and tocols, was utilized as an ingredient (Franke et al., 2010). The content of total carotenoids and the antioxidant activity were evaluated. Figure 4.4 shows the results for the creams added with the carrot extract in the top row, while the data for the

creams added with the algal extract are reported in the lower row. As expected, all the fortified samples were statistically different from the control formulation for total carotenoids, ABTS, and DPPH values, indicating that the fortification substantially improved the nutritional characteristics.

With respect to the control cream (CC), the sample CA showed approximately a six-fold increase for ABTS, while the data indicated a three-fold increase for DPPH. Regarding the sample CB, the observed values were five-fold and two-fold higher for ABTS and DPPH, respectively. These trends were observed also for CD and CE. It is worth noting that formulations with low amounts of added extract (CB and CE) showed values significantly lower than those recorded for the formulation at 28 % of the extract, but not as low as expected, considering the relative proportion of the added amounts (12 and 28%). This might be due to an incomplete extraction occurring when preparing the sample for the antioxidant activity tests. Further investigation should be then carried out to elucidate the point.

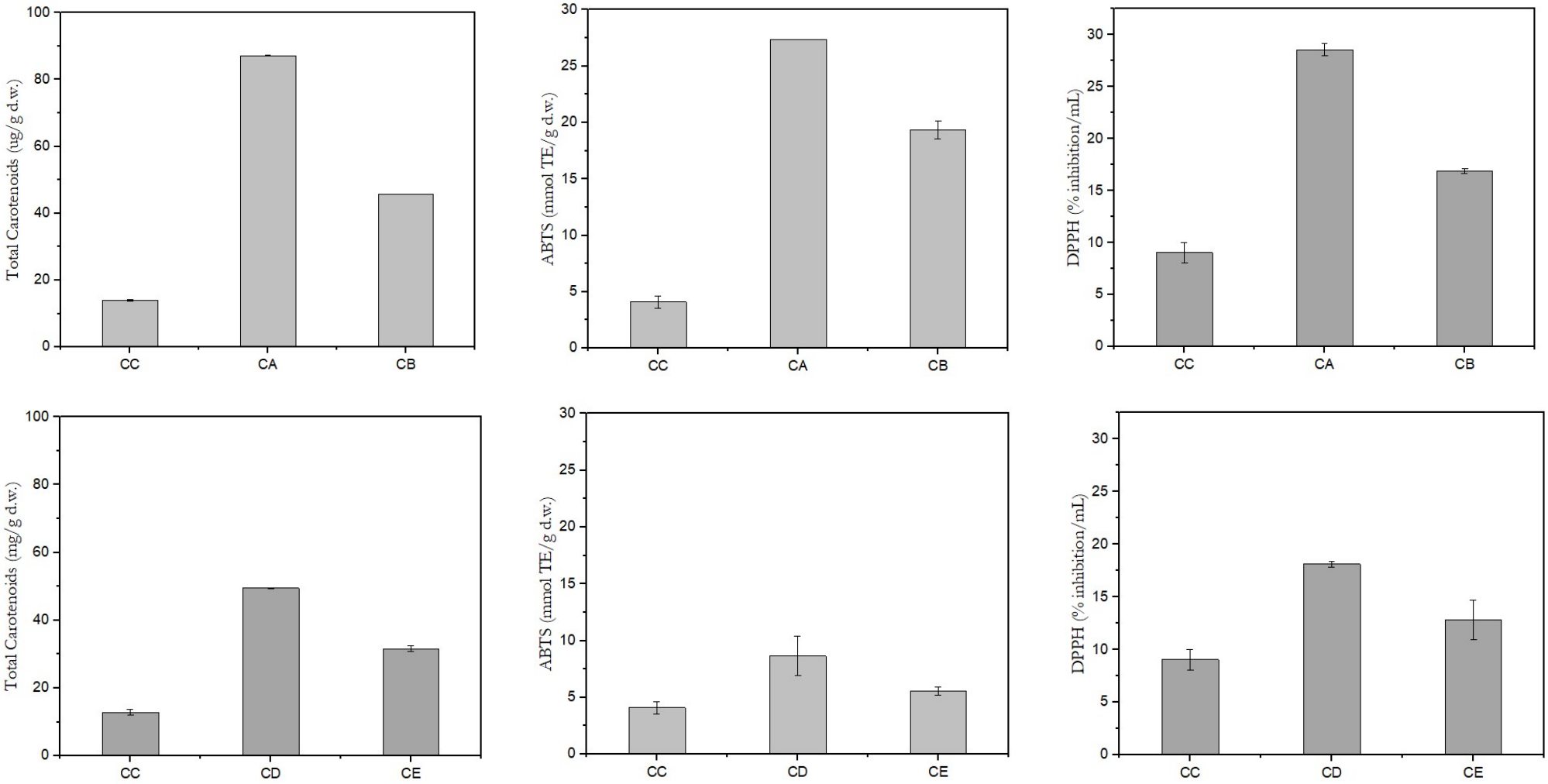

Figure 4.4. Total Carotenoids, ABTS and DPPH values of the base cream (CC), base cream + 28 % carrot peels NaHDES (thymol/DL menthol) extract (CA); base cream + 12 % carrot peels NaHDES (thymol/DL menthol) extract (CB); base cream + 28 % + *Chlorella vulgaris* NaHDES (nonanoic acid/dodecanoic acid) extract (CD); base cream + 12 % + *Chlorella vulgaris* NaHDES (nonanoic acid/dodecanoic acid) extract (CE).

4.4. Conclusions

The results of this study have shown that cocoa and hazelnut spreadable creams fortified with carotenoid-rich NaHDES extracts may be an effective way to enhance the product's nutritional quality and stability. Starting from a commercial cocoa and hazelnut, the addition of two levels of two different NaHDES extracts were investigated. The two types of extracts were obtained using thymol/DL-menthol (1:4) NaHDES on the peels of fresh carrots and nonanoic acid/dodecanoic acid (1:1) on the biomass of the microalga *Chlorella vulgaris*. Furthermore, before evaluating the effect of fortification, the extracts' carotenoid content and antioxidant stability were assessed to acquire information about the preservation role this type of solvent can have towards the extracted biomolecules, which can be then transferred to the fortified products. On the basis of the experimental results, it can be concluded that the addition of the extracts at their low amount is preferable for the impact on the rheological parameters and textural properties. Additionally, considering the preserving effect of thymol/DL-menthol NaHDES on carotenoids and antioxidant activity, its use can represent a significant advantage over the other tested NaHDES extract for the stability of the final fortified product. However, further investigation is needed to evaluate the sensory impact of the added extracts.

As a final consideration, it should be emphasized that much research is still needed before these ready-to-use extracts might find industrial applications for food fortification. Among others, one of the major issues to be addressed is represented by the establishment of Reference Dietary Intake and daily upper limits for NaHDES itself and its starting materials. The redaction of a database with corresponding quantities that could be safely utilized in food items considering NaHDES cytotoxicity, recommendation of daily intake, and sensory properties could indeed significantly foster this application.

4.5. References

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5. General conclusions and future perspectives

In today's food industry, there is an increasing demand for sustainable methodologies in the production of health-promoting natural products. Traditional techniques are no longer considered reliable due to current environmental challenges. In response, the scientific community has established a specialized branch of chemistry called *Green Chemistry*, which focuses on minimizing or eliminating hazardous processes and substances in the industry. One promising and eco-friendly alternative is represented by the use of NaDESs. These solvents are being investigated for several applications in the food sector, including the detection of pesticides, metals, illegal dyes, and packaging contaminants, as well as the extraction of bioactive compounds from food items. NaDESs are generally recognized as safe, causing no harm to users or the environment. The aim of the present PhD project was to investigate the use of hydrophobic NaDESs as a green alternative to traditional organic solvents for recovering bioactive apolar compounds from several food matrices. Acetone was used as the extractant solvent on the same products as a reference.

The investigated extraction process was optimized by implementing BBD and RSM, and identifying the optimal levels of HBA:HBD molar ratio, solvent:sample ratio, and extraction time. The primary focus of the research was to test various NaHDESs for the extraction of carotenoids from several natural sources, namely the processing by-products of fresh carrots, red and yellow peppers, and pumpkins. Eleven NaHDESs composed of carboxylic acids and monoterpenes were prepared and physicochemically characterized, acquiring a significant amount of data lacking in the literature. Afterward, the extraction efficiency of the NaHDESs towards each of the four matrices was assessed, and this step allowed the selection of the most promising solvent to be further investigated for the subsequent optimization phase. The chosen NaDHESs were thymol/DL-menthol (1:1) for the extraction of carrot and yellow pepper peels, thymol/decanoic acid (3:2) for red pepper peels, and DL-menthol/lactic acid (1:2) for pumpkin peels. In all cases, the extraction yields were found to be very similar or better than those achieved using acetone as a solvent at the same extraction conditions.

In the second step of the research, seven other low viscous NaHDESs, composed of fatty acids, were physicochemically characterized and tested for the extraction of carotenoids from the microalga *Chlorella vulgaris*. The screening step allowed the selection of nonanoic/dodecanoic acid (3:1) NaHDES as the

best-extracting media, and when optimizing the process, higher yields could be attained compared with the use of acetone.

Lastly, in order to investigate a direct food application of the carotenoid-rich NaHDES extracts, the fortification of a commercial cocoa and hazelnut spreadable cream was carried out, testing also the antioxidant properties of the extracts, which might find an interesting use in food formulations. Two of the extracts previously optimized for their carotenoid content, namely thymol/DL-menthol (1:4) NaHDES derived from the peels of fresh carrots and nonanoic, acid/dodecanoic acid (1:1) used on the Chlorella vulgaris biomass, were incorporated adding two different amounts for each extract. Afterward, the fortified creams were characterized for their physicochemical and textural properties, carotenoid content, and antioxidant activity. Based on the results obtained, the addition of the extracts at low levels may represent the most suitable choice because of the limited impact on the rheological parameters and textural properties of the creams. Moreover, when considering the use of the NaHDES based on thymol/DL-menthol, this showed a marked action on preserving suitable levels of carotenoids in the extract and improving the antioxidant activity. This may represent a significant advantage over the other tested NaHDES extract when planning food fortification. However, it should be stressed that further investigation is needed to evaluate the sensory impact of the added extracts in the fortified products. As a conclusion remark, it is necessary to mention that the use of NaHDES, before reaching the final consumer, should also be evaluated regarding potential safety issues.

6. List of publications

Results of this PhD thesis have been published in international peer-reviewed journal or are currently under drafting.

The list of all peer-reviewed papers, submitted manuscripts and drafts, oral and poster presentations is given below.

Peer-reviewed papers:

- Sportiello, L., Favati, F., Condelli, N., di Cairano, M., Caruso, M.C., Simonato, B., Tolve, R., Galgano, F. (2023). Hydrophobic deep eutectic solvents in the food sector: Focus on their use for the extraction of bioactive compounds. *Food Chemistry*, 405, 134703.
- Tolve, R., Bianchi, F., Lomuscio, E., Sportiello, L., Simonato, B. (2023). Current Advantages in the Application of Microencapsulation in Functional Bread Development. *Foods*, 12, 96.
- Tolve, R., Tchuenbou-Magaia, F.L., Sportiello, L., Bianchi, F., Radecka, I., Favati, F (2022). Shelf-Life Prediction and Thermodynamic Properties of No Added Sugar Chocolate Spread Fortified with Multiple Micronutrients. *Foods*, *11*, 2358.
- Di Cairano, M., Condelli, N., Cela, N., Sportiello, L., Caruso, M. C., Galgano, F. (2022). Formulation of gluten-free biscuits with reduced glycaemic index: Focus on *in vitro* glucose release, physical and sensory properties. *Lwt*, 154, 112654.
- Di Cairano, M., Tolve, R., Cela, N., Sportiello, L., Scarpa, T., Galgano, F. (2022). "Functional Cereal-Based Bakery Products, Breakfast Cereals, and Pasta Products". In: Punia Bangar, S., Kumar Siroha, A. (eds) *Functional Cereals and Cereal Foods*. Springer, Cham.

Submitted manuscripts and drafts

- Tolve, R., Zanoni, M., Ferrentino, G., Ortega Gonzales, R., Sportiello, L., Scampicchio, M., Favati, F. (2024). Impact of Dietary Fibers on Low-Fact Ice Cream: Physical, Thermal, and Sensory Properties. Submitted.
- Sportiello, L., Tolve, R., Grassi, F., Galgano, F., Zanoni, M., Favati, F. (2023). Ultrasound-assisted extraction of phenolic compounds from Red Radicchio using Natural Deep Eutectic Solvents (NaDESs) for the valorization of agrifood by-products. Drafted.
- Tolve, R., Favati, F., Sportiello, L., A sensory shelf life study for the evaluation of a new eco-sustainable packaging of single-portion croissants. Drafted.
- Sportiello, L., Favati, F., Tolve, R., Giarola, M., Galgano, F. Green Extraction of Carotenoids

from Vegetable by-products Using Natural Hydrophobic Deep Eutectic Solvents. Drafted.

- Sportiello, L., Favati, F., Cazzaniga, S., Galgano, F., Tolve, R.. Green Extraction of Carotenoids from *Chlorella vulgaris* Using Hydrophobic Natural Deep Eutectic Solvents based on Fatty Acids. Drafted.

Oral and poster presentations

- Sportiello, L., Tolve, R., Grassi, F., Galgano, F., Zanoni, M., Favati, F. (2023). Ultrasound-assisted extraction of phenolic compounds from Red Radicchio using Natural Deep Eutectic Solvents (NaDESs) for the valorization of agrifood by-products. *3rd Food Chemistry Conference: Shaping a healthy and sustainable food chain through knowledge*. Dresden Hilton, Germany, 10-12 October. Poster.
- Sportiello, L. (2023). Optimization of the extraction techniques using Deep Eutectic Solvents for the recovery of biomolecules from food and food industry by-products. *27th Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology*, Portici, Università degli Studi di Napoli, 13-15 Settembre. Oral presentation.
- Sportiello, L. (2022). Optimization of the extraction techniques using Deep Eutectic Solvents for the recovery of biomolecules from food industry by-products. *26th Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology*, Asti, Università degli Studi di Torino, 19-21 Settembre, 296-297. ISBN: 9788875902278. Poster.
- Sportiello, L. (2021). Optimization of the extraction techniques using Deep Eutectic Solvents for the recovery of biomolecules from food industry by-products. *First Virtual Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology*, Palermo, Università degli Studi di Palermo, 14-15 Settembre. Poster.