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Exploring the use of natural deep eutectic solvents (NaDES) as a green strategy to extract *Schizochytrium* sp. lipids

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Abstract

Marine oleaginous microorganisms such as *Schizochytrium sp.* represent a sustainable source of polyunsaturated fatty acids, particularly docosahexaenoic acid (DHA), but their efficient and environmentally compatible extraction remains challenging. This study evaluates natural deep eutectic solvents (NaDES) as green alternatives for lipid extraction, with a particular focus on extraction efficiency, recoverability, and biological safety. A menthol:thymol (Me:Th) NaDES was first optimized using ultrasound-assisted extraction (UAE) combined with response surface methodology. Probe-assisted UAE achieved a maximum predicted yield of 61.3 mg FA g⁻¹ extract under optimized conditions (10 min, 86% amplitude, 100% pulse), which was experimentally validated at 54.32 ± 1.73 mg FA g⁻¹ extract. Despite efficient lipid solubilization, phase separation between NaDES and extract was not feasible. To address this limitation, the optimized UAE conditions were transferred to four hydrophobic fatty-acid-based NaDES systems (C8:C12, C8:Me, C8:Th, C8:Ca). In contrast to Me:Th, these systems enabled efficient lipid recovery via reversible hydrophobicity switching using Jeffamine. Among the tested solvents, C8:C12 showed the best overall performance in terms of extraction yield, fatty acid content, and compositional similarity to Soxhlet extracts. Cytotoxicity assessment using HCEC-1CT and HCT116 cells (CTB and SRB assays) demonstrated no significant reduction in cell viability for NaDES or derived extracts at concentrations up to 100 µg mL⁻¹. Overall, this study demonstrates that fatty-acid-based NaDES, in combination with switchable hydrophobicity strategies, enable efficient extraction and recovery of lipids from *Schizochytrium sp.*, providing a promising and more sustainable alternative to conventional solvent-based processes.

Zusammenfassung

Marine oleaginöse Mikroorganismen wie *Schizochytrium sp.* stellen eine nachhaltige Quelle für mehrfach ungesättigte Fettsäuren dar, insbesondere für Docosahexaensäure (DHA), jedoch bleibt deren effiziente und umweltverträgliche Extraktion eine Herausforderung. In dieser Arbeit wurden Natural Deep Eutectic Solvents (NaDES) als grüne Alternativen zur Lipidextraktion untersucht, mit besonderem Fokus auf Extraktionseffizienz, Rückgewinnbarkeit und biologische Sicherheit. Zunächst wurde ein Menthol:Thymol-(Me:Th)-NaDES mittels ultraschallunterstützter Extraktion (UAE) in Kombination mit der Response-Surface-Methodik optimiert. Die sondenbasierte UAE erreichte unter optimierten Bedingungen (10 min, 86 % Amplitude, 100 % Puls) eine maximale vorhergesagte Ausbeute von 61,3 mg FS g⁻¹ Extrakt, welche experimentell mit 54,32 ± 1,73 mg FS g⁻¹ Extrakt bestätigt wurde. Trotz effizienter Lipidsolubilisierung war jedoch keine Phasentrennung zwischen NaDES und Extrakt möglich. Zur Überwindung dieser Limitation wurden die optimierten UAE-Bedingungen auf vier hydrophobe, fettsäurebasierte NaDES-Systeme (C8:C12, C8:Me, C8:Th, C8:Ca) übertragen. Im Gegensatz zu Me:Th ermöglichten diese Systeme eine effiziente Lipidrückgewinnung über einen reversiblen Wechsel der Hydrophobizität unter Verwendung von Jeffamine. Unter den getesteten Lösungsmitteln zeigte C8:C12 die beste Gesamtleistung hinsichtlich Extraktionsausbeute, Fettsäuregehalt und Zusammensetzungsähnlichkeit zu Soxhlet-Extrakten. Die Untersuchung der Zytotoxizität mittels HCEC-1CT- und HCT116-Zellen (CTB- und SRB-Assays) zeigte keine signifikante Verringerung der Zellviabilität für NaDES oder daraus gewonnene Extrakte bei Konzentrationen bis 100 µg mL⁻¹. Insgesamt zeigt diese Arbeit, dass fettsäurebasierte NaDES in Kombination mit Strategien der schaltbaren Hydrophobizität eine effiziente Extraktion und Rückgewinnung von Lipiden aus *Schizochytrium sp.* ermöglichen und damit eine vielversprechende sowie nachhaltigere Alternative zu konventionellen lösungsmittelbasierten Prozessen darstellen.

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List of Abbreviations

ANOVA	Analysis of variance
BBD	Box–Behnken Design
C12	Lauric/Dodecanoic acid
C8	Caprylic/Octanoic acid
Ca	Carvacrol
CTB	CellTiter-Blue assay
DHA	Docosahexaenoic acid
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPA	Docosapentaenoic acid
EGF	Epidermal Growth Factor
EPA	Eicosapentaenoic acid
FA	Fatty Acid
FAME	Fatty Acid Methyl Ester
FCS	Fetal Calf Serum
FFA	Free Fatty Acid
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
IS	Internal Standard
Me	Menthol
NaDES	Natural Deep Eutectic Solvent(s)
PUFA	Polyunsaturated Fatty Acid
RSM	Response Surface Methodology
SRB	Sulforhodamine B assay
TAG	Triacylglycerol
UAE	Ultrasound-Assisted Extraction

1 Introduction

In recent years, microalgae and other marine oleaginous microorganism have emerged as a promising and sustainable source of bioactive lipids, including both neutral lipids such as essential fatty acids and triacylglycerols (TAGs) as well as polar lipids such as glycolipids and phospholipids. These compounds are of increasing interest in the food, pharmaceutical, and biofuel industries due to their nutritional value and functional properties. However, the extraction of these lipids remains a major challenge, particularly due to the structural complexity of the algal cell wall and the need for environmentally friendly extraction techniques [1], [2].

Conventional lipid extraction methods, such as Soxhlet or Folch extraction, typically rely on large volumes of toxic organic solvents, which are not only hazardous to human health and the environment but also energy-intensive. Therefore, the development of alternative, greener extraction approaches has become a key focus [3].

Natural deep eutectic solvents (NaDES) have recently gained attention as promising alternatives to conventional solvents [4]. Formed from mixtures of hydrogen bond donors and acceptors, NaDES exhibit tunable physicochemical properties, low toxicity, and biodegradability. These characteristics make them attractive candidates for the selective extraction of both polar and non-polar biomolecules from natural sources [5]. Nonetheless, the application of NaDES in microalgal lipid extraction is still relatively underexplored. To date, only few studies have applied NaDES to marine oleaginous microorganisms lipid extraction, with most work focusing on polar compounds.

This Master Thesis will investigate the use of natural deep eutectic solvents as sustainable alternatives for the extraction of non-polar lipids from *Schizochytrium sp.* Biomass a marine oleaginous microorganism. The study will focus on ultrasound-assisted extraction using different NaDES combinations and will compare their efficiency and environmental performance to conventional solvent-based methods. In addition, various strategies for recovering the extracted lipids from the NaDES phase will be explored, addressing one of the main challenges associated with these systems. Selected extracts will also be evaluated for their potential cytotoxic effects to assess their suitability for applications in the food or pharmaceutical industries.

2 Theoretical Background

2.1 Lipids in microalgae and marine oleaginous microorganisms

Microalgae and other marine oleaginous microorganisms represent a phylogenetically diverse group of unicellular organisms capable of converting sunlight, carbon dioxide, and inorganic nutrients into a wide variety of bioactive compounds [6]. Their rapid growth, simple nutritional requirements, and ability to thrive in non-arable environments make them a highly promising and sustainable feedstock for biotechnological applications [6], [7], [8]. Over the past decades, microalgae and related lipid-accumulating microorganisms have gained increasing attention as renewable sources of valuable metabolites such as pigments, proteins, polysaccharides and lipids [8]. These lipids play central roles not only in cellular physiology but also as precursors for nutraceutical, pharmaceutical, and biofuel products [9]. Microalgae are particularly rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), serving as natural sources of omega-3 fatty acids for dietary supplements, anti-inflammatory agents, and functional food ingredients [10].

The lipid fraction of these oleaginous microorganisms is chemically and functionally heterogeneous. Depending on the species, cultivation conditions, and stress factors [1], [11], the average lipid content varies between 1 and 70 % of the cell's dry weight, but under certain conditions some species can reach 90% of dry weight [11], [12]. These lipids also vary in their degree of saturation, chain length, and polarity [11]. Broadly, these lipids can be categorized into structural lipids (polar) and storage lipids (neutral and nonpolar) [1], [11]. Neutral lipids, including triacylglycerols (TAGs), wax esters, and sterol esters, primarily function as energy storage molecules and are localized within cytosolic lipid droplets. TAGs consist of three fatty acid chains esterified to a glycerol backbone. In contrast, polar lipids, such as phospholipids and glycolipids, are integral components of cellular membranes, where they contribute to membrane structure, integrity, and fluidity [1].

The chemical form of the fatty acids, whether esterified (TAGs, phospholipids) or free (free fatty acids, FFAs), strongly influences both solubility and extractability [1], [11]. Free fatty acids can participate in hydrogen bonding through their carboxylic groups, resulting in higher polarity compared to esterified analogues. Consequently, solvent selectivity in extraction depends largely on polarity matching: non-polar solvents such as hexane or hydrophobic NaDES preferentially dissolve neutral lipids (TAGs), whereas polar or protic solvents favor the extraction of membrane-bound lipids and FFAs [11]. Understanding these polarity-dependent interactions is crucial when designing alternative solvent systems

like NaDES, whose physicochemical properties can be tuned via the composition of hydrogen bond donors (HBDs) and acceptors (HBAs) to target specific lipid classes selectively [13].

Another important determinant of extractability is the structural complexity of the algal cell wall. Many microalgae possess multilayered cell walls composed of polysaccharides, glycoproteins, and aliphatic polymers that form strong physical barriers against solvent penetration [1]. The resulting diffusion limitations significantly influence extraction kinetics and necessitate mechanical or assisted disruption techniques to access intracellular lipid reservoirs. These matrix effects, combined with the chemical diversity of lipids, underline the importance of designing extraction systems that can effectively penetrate the biomass while maintaining selectivity toward target lipid classes [1], [11].

2.1.1 *Schizochytrium sp.*

Schizochytrium sp., a heterotrophic marine microorganism belonging to the class *labyrinthulomycetes*, has emerged as one of the most promising alternative sources for polyunsaturated fatty acids (PUFAs) over the past two decades [14]. Its exceptional capacity to accumulate high levels of lipids, particularly rich in omega-3 fatty acids, makes it a prime candidate for industrial biotechnology and a sustainable alternative to traditional fishery resources [15]. Oils derived from this organism are authorized as a novel food in the European Union, where “DHA- and EPA-rich oil from the microalgae *Schizochytrium sp.*” is approved for use in a wide range of food categories [16]. This regulatory status reflects the established safety and widespread use of *Schizochytrium*-derived oils in foods, supplements, and specialized nutritional products.

Schizochytrium sp. is classified as an oleaginous microorganism, capable of accumulating lipids up to 50% of its dry cell weight under optimized conditions [17]. The lipid profile is notably characterized by a very high proportion of long-chain polyunsaturated fatty acids, strongly dominated by docosahexaenoic acid (DHA), which typically accounts for around 40 % of total fatty acids [12], [18], [19]. DHA is important because it has significant effects on human health, such as the development of brain function and central nervous system in infants and maintenance of normal brain function in adults [20] and helps to prevent diseases like cardiovascular disease, diabetes type II and inflammation [10] among other health benefits. Additional components include docosapentaenoic acid (DPA n-6) and eicosapentaenoic acid (EPA) as well as myristic (14:0) and palmitic acid (16:0), forming a characteristic composition to further enhance its nutritional value [5, 7].

The combination of lipid diversity, variable polarity, and cell wall resistance highlights the central challenge in lipid recovery: developing solvent systems that are not only efficient and selective but also environmentally sustainable. These requirements provide the conceptual foundation for exploring natural deep eutectic solvents as a green alternative to conventional organic solvents, which will be discussed in the following section.

2.2 Natural Deep Eutectic Solvents (NaDES)

2.2.1 Definition and formation principles

Abbott et al. (2003) defined deep eutectic solvents (DES) as liquid mixtures whose melting point is reduced by more than 100 °C relative to the melting points of the individual components. The first described DES consisted of choline chloride and urea, forming a liquid at 12 °C despite the much higher melting temperatures of the pure substances (302 °C and 133 °C, respectively) [22]. Deep eutectic solvents are formed by mixing two or more components, typically a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), at a specific molar ratio. Upon mixing, these components interact through hydrogen bonding and other non-covalent forces, resulting in a significant depression of the melting point compared to the individual compounds [23], [24].

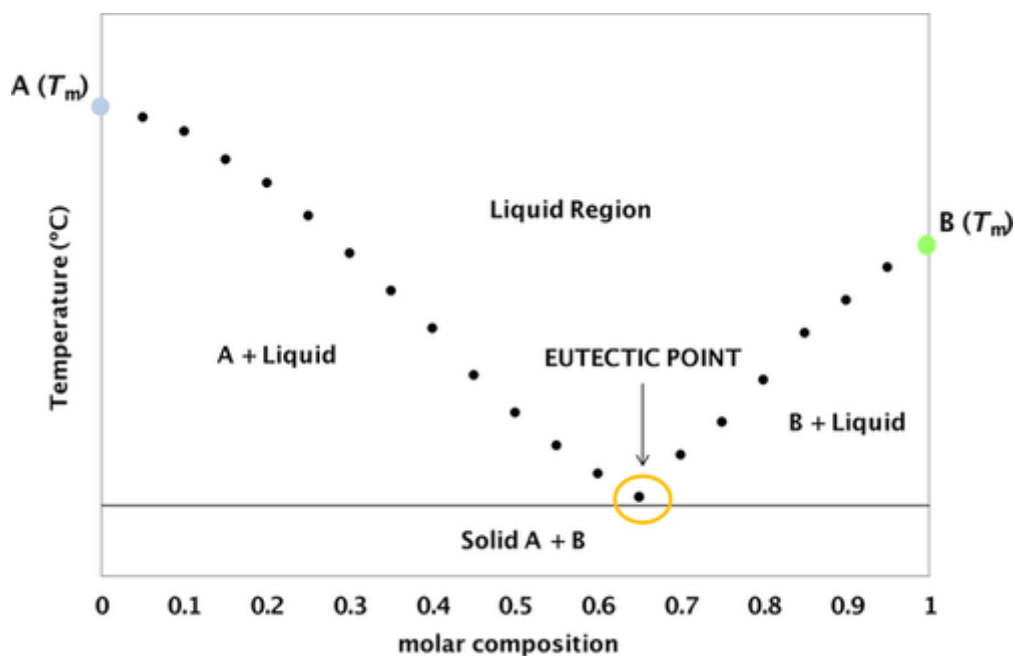


Figure 1: Schematic phase diagram of the melting temperatures of different mixtures [4].

Figure 1 shows how combining two solid components at specific molar ratios leads to a deep eutectic point, characterized by a melting temperature far lower than that of either pure compound. The liquid region spans a broad composition range, indicating that DES formation does not rely on a single precise molar ratio but can be tuned by adjusting temperature and composition [4].

The fundamental principles of DES formation, melting-point depression, hydrogen-bond networking, and tunable polarity, also apply to systems composed entirely of natural metabolites. Such mixtures are classified as Natural Deep Eutectic Solvents. Unlike conventional organic solvents or ionic liquids, NaDES are composed of naturally occurring, biodegradable, and low-toxicity compounds such as organic acids, amino acids, sugars, polyols, or terpenes, which are often inexpensive and easily accessible [24]. These features make them particularly attractive for applications in green chemistry, bioprocessing, and pharmaceutical formulations [25].

2.2.2 Classification of NaDES

NaDES can be classified into five main types according to the chemical nature of their components and the interactions responsible for their eutectic behavior [26]. Type I systems consist of quaternary ammonium salts and metal salts; Type II combine quaternary ammonium salts with metal chlorides; Type III contain a quaternary ammonium salt as HBA and a molecular HBD such as an acid, alcohol, or amide; Type IV include metal salts with molecular HBDs; and Type V consist entirely of neutral organic molecules without ionic species and are based on phenolic hydrogen bonds [4]

2.2.3 Hydrophobic Natural Deep Eutectic Solvents

Van Osch et al. reported in 2015 a class of hydrophobic deep eutectic solvents composed of capric acid and various quaternary ammonium salts [27]. Type V NaDES represent a distinct subgroup of eutectic systems composed exclusively of neutral organic molecules. These systems typically consist of menthol- or thymol-based mixtures combined with sugars, polyols, fatty acids, or various short-chain fatty acid pairs [4], [26], [28]. Because of their reduced polarity, hydrophobic NaDES display high selectivity toward neutral lipid classes, particularly triacylglycerols, while the extraction of polar lipids such as phospholipids is intrinsically limited. This selectivity is advantageous when targeting non-polar lipid fractions, as it minimizes the co-extraction of membrane-bound components [28].

Overall, hydrophobic NaDES combine the characteristic benefits of NaDES, simple preparation, low cost, compositional tunability, and low volatility, with a hydrophobic profile that enables efficient extraction of non-polar analytes from aqueous matrices. Their immiscibility with water, strong hydrogen-bond-driven structural stability, and often favorable toxicological and environmental properties position them as promising alternatives to conventional hydrophobic organic solvents in natural product extraction, food analysis, and separation science. Nonetheless, challenges such as elevated viscosity, variations in phase separation behavior, and the need for more comprehensive toxicological evaluation remain important considerations for future development.

2.2.4 *Preparation of NaDES*

One of the main advantages of NaDES is their simple preparation. They are formed by directly mixing a hydrogen-bond donor with a hydrogen-bond acceptor, and the entire amount of starting material becomes part of the final solvent, without generating by-products. Heating is the most commonly used method to promote the formation of the eutectic mixture, although other approaches such as mechanical grinding or dissolving the components in a small volume of water followed by evaporation have also been reported. [23]. An important limitation of the heating method is the low thermal stability of many natural compounds, which can lead to the formation of unwanted degradation products if excessive temperatures are applied. [29].

2.2.5 *Physicochemical properties of hydrophobic NaDES*

Properties such as density, viscosity, and melting point are critical parameters that determine the practical usability of DES and influence their extraction performance [30]. A major advantage of these tunable solvents lies in the ability to adjust their composition, allowing mixtures with tailored physicochemical characteristics. Among all parameters, the chemical nature and molar ratio of the HBD and HBA play the dominant role in defining their final properties [31].

2.2.5.1 Density

Density is one of the most important parameters influencing phase separation in liquid-liquid extraction [32]. Reported densities of hydrophobic NaDES span a broad range ($0.85\text{--}1.5\text{ g cm}^{-3}$), largely reflecting the densities of their components with tendencies to an increase compared to its components, due to the strong interactions between them [33], [34]. The density of eutectic mixtures are evidently influenced by several factors, including the characteristics of its constituents, their molar ratio, the

temperature, and the presence of water [32]. For practical extraction, NaDES with densities substantially different from that of water are preferred, as similar densities can lead to prolonged separation times or emulsion formation. Consequently, substantial density differences enhance phase separation efficiency [30]. NaDES density plays a crucial role in phase separation and extraction efficiency. Matching the density of DES with that of the target compound can enhance extraction efficiency [34].

2.2.5.2 Viscosity

Viscosity is another critical physical property of NaDES. It is a decisive factor for mass transfer, mixing behavior, and overall extraction efficiency [30]. In NaDES, viscosity is driven primarily by the extent and strength of the hydrogen-bonding network, which restricts molecular mobility [31]. Hydrophobic NaDES generally exhibit lower viscosities than hydrophilic DESs because they lack strong Coulombic interactions. Nevertheless, many hydrophobic NaDES still possess moderately high viscosities that may hinder rapid extraction. Strategies applied to reduce the viscosity of NaDES include preheating and water addition. However, adding water may disrupt the hydrogen-bonding network within NaDES, whereas heating may result in solvent degradation [29], [30].

The viscosity of NaDES is significantly influenced by their constituent components. Viscosity increases with increasing alkyl chain length in tetraalkylammonium HBAs, reflecting stronger hydrophobic interactions and reduced molecular mobility. In contrast, long-branched chains can reduce viscosity by disrupting packing. Across different HBA classes, viscosity typically follows: quaternary ammonium salts > menthol > thymol $\approx$ fatty acids [4].

2.2.5.3 Melting Point

A defining feature of NaDES is that their melting points are substantially lower than those of their individual components, allowing them to be liquid under ambient conditions [30]. From a molecular viewpoint, the melting point of NaDES is governed mainly by hydrogen-bonding interactions and van der Waals forces between the HBD and HBA. Stronger hydrogen bonds lead to tighter intermolecular packing, greater structural stability, and therefore a higher melting point [34]. Their strength depends on the polarity and functional groups of the components; highly polar molecules or HBDs with multiple hydroxyl or amino groups can form more extensive hydrogen-bond networks. For instance, phenol and ethanolamine form multiple hydrogen bonds, resulting in an increased melting point in the corresponding NaDES [35].

Additional factors such as the component ratio and the alkyl chain structure also influence the melting point of NaDES. An optimal molar ratio promotes the formation of a well-defined hydrogen-bond network, while increasing alkyl chain length in fatty acids generally leads to higher melting points. Consequently, achieving a target melting point requires careful tuning of component structure, polarity, molar ratio, and steric effects to ensure that the NaDES remains liquid and operationally stable under typical extraction conditions [36].

2.2.6 Lipid Recovery Strategies

One of the main challenges in NaDES-based extraction processes is the recovery of the extract without the solvent as well as to be able to recycle the solvent. Unlike volatile organic solvents, NaDES cannot be separated from the solute by simple evaporation due to their negligible vapor pressure and thermal stability [28]. This non-volatility, while advantageous for safety and environmental reasons, complicates product isolation and requires the use of alternative recovery strategies [37]. Because NaDES are multi-component systems stabilized by extensive intermolecular hydrogen bonding, their chemical composition and intermolecular structure are inherently more complex than those of conventional solvents. As a result, no universal recovery method exists; instead, the specific strategy and operational conditions must be selected with regard to the physicochemical properties of the NaDES, the extraction system, and the nature of coexisting substances [38]. Therefore, alternative strategies are required that enable the selective separation of lipids while preserving the structural integrity of both the NaDES and the target compounds.

Several recovery approaches have been proposed to overcome this limitation, but not all are effective for hydrophobic NaDES systems. One common strategy is antisolvent precipitation, in which a polar solvent such as methanol, ethanol, or water is added to the NaDES-lipid mixture [39], [40]. The reduction in solvent polarity decreases lipid solubility, promoting precipitation or phase separation [37]. However, in viscous hydrophobic NaDES, this method frequently leads to the formation of stable emulsions and requires additional purification steps, making it unsuitable for efficient lipid release. Similar limitations have been reported in the recovery of flavonoids and curcuminoids using antisolvent precipitation, where only moderate recoveries were achieved [41].

A second approach relies on temperature-induced phase separation, where cooling or freezing promotes demixing of the NaDES and lipid phases. At lower temperatures, the solubility of lipids in the eutectic matrix decreases due to reduced molecular motion and weakened hydrogen-bond

interactions, facilitating lipid aggregation and separation. However, this method can be slow and may not achieve complete phase separation, especially for highly viscous NaDES [42].

A third potential method is evaporation-assisted separation, in which NaDES are heated under reduced pressure for extended time periods to promote lipid layering. However, due to the negligible vapor pressure of NaDES, effective solvent removal requires evaporation at 70 °C for approximately 20 hours, which contradicts the principles of green chemistry [43].

A more advanced and versatile method for lipid recovery involves switchable hydrophobicity, a concept based on the reversible modification of solvent polarity [38]. In general, NaDES can undergo polarity changes when triggered by physical or chemical stimuli such as pH, CO₂, temperature, or specific reactants, allowing controlled switching between hydrophobic and hydrophilic states. Such phase-switching systems have gained attention because they allow separation and solvent recovery without relying on evaporation or energy-intensive processing. [38]

This strategy has been successfully demonstrated using amine-based systems such as Jeffamine, a polyetheramine that can reversibly alter the hydrophilicity of the system through protonation and deprotonation of the hydrogen bond acceptor. Upon the addition of an acidic solution (e.g., HCl or CO₂-saturated water), the amine groups of Jeffamine become protonated, converting the system from hydrophobic to hydrophilic. This polarity shift allows phase separation, in which the lipid fraction migrates into a distinct nonpolar layer, effectively releasing it from the NaDES matrix. When the pH is subsequently increased, Jeffamine returns to its deprotonated, hydrophobic form, enabling regeneration of the original solvent properties. The reversibility of this process is critical, as it allows repeated cycles of lipid extraction and solvent recovery without significant loss of efficiency [44].

From a sustainability perspective, an efficient recovery strategy should minimize energy input, solvent waste, and environmental impact while maintaining high selectivity for the target lipids. In contrast to traditional solvent evaporation or distillation, which require substantial thermal energy and generate volatile emissions, the polarity-switching method operates under mild conditions and can be performed at ambient temperature and pressure [38]. The recyclability of NaDES and Jeffamine further reduces material consumption and waste generation, contributing to improved life-cycle performance. The development of efficient and sustainable recovery methods remains one of the key challenges for implementing NaDES-based extraction on a larger scale. The ability to reversibly modify solvent

polarity through chemical or physical triggers provides a promising route toward closed-loop extraction systems, where both solvent and product can be reused with minimal environmental footprint.

2.3 Extraction methods

The extraction and analysis of lipids and fatty acids from microalgae and marine oleaginous microorganism differ substantially from those of other biological matrices such as vegetable oils because they possess rigid cell walls and exhibit a high diversity of lipid classes and fatty acid compositions [1]. Because microalgae contain a wide variety of lipid classes with different polarities, the amount of lipid extracted strongly depends on the solvent system used. This variation is one of the main reasons for inconsistencies in lipid yields and reported fatty-acid profiles across studies [1], [2], [45]. Efficient extraction therefore requires methods capable of disrupting the cell wall to release intracellular lipids. Conventional protocols such as the Soxhlet [46] and Folch [47] methods use hexane or chloroform-methanol mixtures to solubilize the full spectrum of lipid classes, but these procedures are difficult to scale up.

An extraction method should be rapid, efficient, and mild enough to prevent lipid degradation while remaining economically viable [48]. In practice, extraction begins with mechanical or physicochemical disruption of the cell wall, followed by solvent-based recovery of the released lipids. Effective cell disruption is therefore a prerequisite for high extraction yields [45]. Numerous studies have focused on optimizing disruption and extraction strategies to increase lipid recovery while reducing costs [49]. The most frequently applied cell wall disruption methods include Ultrasound, Microwave, Osmotic shock, Enzymatic breakdown and Pressing, followed by extraction methods including Organic solvents, Super critical fluid and Soxhlet [45], [49], [50].

2.3.1 Conventional Extraction Methods (Soxhlet Extraction)

The Soxhlet extraction is one of the oldest and most established techniques for isolating lipids and other non-polar compounds from biological materials [46]. The method is based on the continuous circulation of a solvent that repeatedly evaporates, condenses, and percolates through the sample. This continuous reflux allows nearly complete extraction of soluble components and provides high reproducibility, which is why Soxhlet extraction is commonly used as a reference method in lipid research. However, the technique requires long extraction times, large solvent volumes, and high energy input for heating and solvent recovery. Furthermore, it offers no selectivity for different lipid classes

and relies on volatile, toxic organic solvents such as n-hexane. These disadvantages make Soxhlet extraction environmentally unsustainable and highlight the need for greener alternatives. In this context, the following section introduces ultrasound-assisted extraction as a more efficient and sustainable technique suitable for integration with NaDES.

2.3.2 *Ultrasound-Assisted Extraction (UAE)*

Ultrasound has gained considerable attention as a green and cost-effective method for the extraction of natural products. UAE enhances the release of intracellular compounds by applying acoustic energy to the solvent-solid system. When ultrasonic waves pass through a liquid, alternating compression and rarefaction cycles generate cavitation bubbles that violently collapse, producing shock waves, microjets, microturbulence, and microstreaming. These mechanical effects intensify mass transfer, facilitate solvent penetration, and promote rapid disruption of cellular structures, enabling efficient release of target compounds [3], [41].

The extraction performance of UAE depends on several operational factors, including the choice of solvent, sonication amplitude, time, pulse mode, and temperature, as well as characteristics of the sample such as matrix structure and particle size [51], [52]. Ultrasound can be applied either via bath systems or via ultrasonic probes. Probes deliver a more focused and uniform energy input and allow stricter control over critical parameters, enabling higher reproducibility and more efficient cavitation compared to bath configurations [51], [52], [53].

Overall, UAE provides a rapid, energy-efficient extraction technique that increases solvent-solid contact area and accelerates solute diffusion without requiring dispersive solvents. It has therefore become a widely used tool in green extraction processes [41].

2.3.2.1 Synergistic action of NaDES and ultrasound

The combination of NaDES and ultrasound offers a highly effective and sustainable intensification strategy for the extraction of bioactive compounds. When ultrasound is applied to NaDES-based systems, cavitation significantly enhances mass transfer and accelerates the rupture of cellular structures. Ultrasonic waves induce alternating compression and rarefaction cycles, during which cavitation bubbles form, expand, and collapse. Their implosion generates localized hotspots with high pressures and temperatures, producing intense mechanical effects that cause cell wall disruption, particle size reduction, and enhanced solvent penetration [54]. These effects complement the chemical solvation ability

of NaDES: while ultrasound provides physical disintegration of the biomass, NaDES selectively solubilize and stabilize the released intracellular compounds through their hydrogen-bond networks and polarity characteristics [55].

The physicochemical properties of NaDES, particularly viscosity, influence cavitation behavior. Low-viscosity NaDES better transmit acoustic energy and support more efficient bubble dynamics, whereas highly viscous systems dampen cavitation and reduce mass transfer [56]. Thus, optimal performance depends on balancing NaDES composition, ultrasound amplitude and frequency, solvent-to-solid ratio, and extraction temperature [55].

2.4 Cell Culture and Cytotoxicity Testing

For NaDES to be suitable for applications in food, nutraceutical, or pharmaceutical formulations, their biological safety must be ensured. Although composed of natural and generally recognized compounds, when combined into eutectic mixtures, individual components may interact in ways that modify polarity, hydrogen-bonding patterns, and membrane affinity, potentially giving rise to emergent biological effects that differ from those of the pure substances [57]. Therefore, each NaDES must be evaluated individually to verify that chemical sustainability is accompanied by biological compatibility.

Cytotoxicity refers to the ability of a substance to impair cellular viability, proliferation, or metabolic function. Such effects typically arise from disturbances of membrane integrity, mitochondrial dysfunction, oxidative stress, or interference with essential biochemical pathways. *In vitro* cytotoxicity assays are widely used to evaluate the biocompatibility of solvents, extracts, or chemical components, as they provide a controlled environment to quantify cellular responses to defined concentrations of test substances [58].

Two complementary assay principles are commonly employed to determine cytotoxicity: metabolic activity and biomass determination. The CellTiter-Blue (CTB) assay measures the reduction of resazurin to the fluorescent product resorufin by mitochondrial and cytosolic enzymes, providing an indirect but sensitive indicator of metabolic activity [59]. A decrease in fluorescence reflects reduced cellular metabolism and is therefore interpreted as decreased viability. In contrast, the Sulforhodamine B (SRB) assay quantifies total cellular protein content by staining basic amino acid residues under mildly acidic conditions. Since protein mass correlates with cell number, SRB provides a robust measure of cell biomass and proliferation [60]. Together, these assays capture two distinct dimensions of cytotoxicity,

metabolic competency and total cell mass, allowing a more comprehensive evaluation of cellular responses to NaDES, their components, and derived extracts.

3 Objectives

The general objective is to evaluate the potential of different NaDES as a green extraction solvent for recovering lipids from *Schizochytrium sp.* through ultrasound-assisted extraction and to compare their performance and safety with conventional solvent systems.

Specific objectives

- To optimize the ultrasound-assisted extraction parameters of a menthol-thymol NaDES using response surface methodology in both ultrasonic bath and probe systems and to identify the key factors influencing lipid yield.
- To assess the environmental sustainability of the developed methods using quantitative green metrics (AGREEprep) and benchmark them against conventional solvent extraction.
- To extend the optimized and greener extraction protocol to fatty-acid-based NaDES (C8:C12, C8:Th, C8:Me, C8:Ca) and compare their extraction efficiency.
- To investigate lipid recovery strategies and NaDES separation based on reversible hydrophobicity switching using Jeffamine D-230 and to assess their potential for solvent recycling.
- To evaluate the cytotoxicity of NaDES and NaDES-derived lipid extracts in human epithelial (HCEC-1CT) and colorectal carcinoma (HCT116) cells to ensure their compatibility for potential food and pharmaceutical applications.

Together, these objectives provide a structured framework linking solvent design, extraction efficiency, lipid selectivity, and cytotoxicity assessment into a coherent evaluation of NaDES as a green alternative for lipid extraction from *Schizochytrium sp.*

4 Materials and Methods

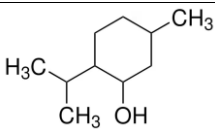
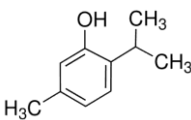
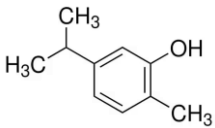
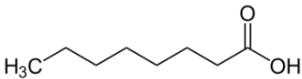
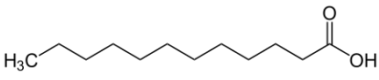
4.1 NaDES extraction, recovery and chemical analysis

4.1.1 Chemicals and Materials

The biomass of *Schizochytrium sp.* (batch AKEY_0165) was purchased from Algald SL (Madrid, Spain). *Schizochytrium sp.* commercial oil was acquired from Nutilab S.L. (Valencia, Spain). Both the biomass and the reference oil were stored at low temperature and protected from light to prevent oxidative degradation.

Menthol, thymol, carvacrol, caprylic acid (C8), and lauric acid (C12) were used as hydrogen bond donors (HBD) and/or acceptors (HBA) for NaDES preparation. All compounds were purchased from Sigma-Aldrich (Schnelldorf, Germany) and used without further purification. Their structural formulas and key physicochemical properties are summarized in Table 1.

Table 1: Chemical structures and key properties of the compounds¹ used for the preparation of NaDES in this study.

Compound	Structure	Purity	HBA/HBD ²	molecular weight (g mol ⁻¹)
DL-Menthol (Me)		≥95 %	HBA/HBD	156.27
Thymol (Th)		≥99 %	HBD	150.22
Carvacrol (Ca)		≥99 %	HBD	150.22
Octanoic acid (C8)		≥98 %	HBA/HBD	144.21
Lauric acid (C12)		≥98 %	HBA/HBD	200.32

¹ Data taken from PubChem [61] and SigmaAldrich [62]

² Hydrogen bond acceptor/Hydrogen bond donor

Methyl tricosanoate (analytical standard, $\geq 99\%$) was used as internal standard (IS) for GC-MS analysis. For the reversal of hydrophobicity, a Jeffamine D-230 solution (poly(propylene glycol) bis(2-aminopropyl ether)) was used. Both purchased from Sigma-Aldrich (Schnelldorf, Germany). Sodium sulfate was provided by Lab-Scan Analytical Sciences (Gliwice, Poland). Hexane was supplied by Macron Fine Chemicals (Center Valley, PA, USA). All chemicals were used as received.

4.1.2 *Preparation and Characterization of Me:Th NaDES*

NaDES were prepared by weighing the first compound on an analytical balance (Sartorius Extend ED224S, readability: 0.1 mg, maximum capacity: 220 g) directly into flask with a magnetic stirrer already inserted. The second compound is weighed into a weighing boat made of aluminum foil and transferred into the flask. The mixture is put into a water bath at 80 °C and stirred for 30 minutes. After a clear liquid is obtained, the NaDES is left to cool and stored at room temperature.

4.1.2.1 Density

Due to the viscosity of the NaDES, it is not possible to ensure the complete release of the volume. Instead, the density was determined by pre-weighing the tip of a 1 mL Eppendorf pipette, subsequently, 1 mL of the sample was aspirated into the tip, and the loaded tip was re-weighed to obtain the combined mass. The mass of the NaDES was calculated by subtracting the tare mass of the empty tip from the loaded-tip mass. The measurement was carried out at room temperature and the density is expressed in g/mL.

It was tested if the preparation time influences the density by taking aliquots at set points during the production of Me:Th (15, 30, 45 minutes and 1, 2, 3, 4, 5, 6 and 16 hours) and measuring the density as described above. The density of the NaDES remained constant over the course of the preparation time, indicating that the mixing and heating process reached completion within the first 15 minutes.

4.1.3 *Ultrasound Assisted Extraction (UAE)*

For the UAE, the samples were subjected to two different treatments: an ultrasonic bath and an ultrasonic probe. Both methods rely on piezoelectric transducers as sources of ultrasound energy. The probe-based systems provide higher ultrasonic intensity, while ultrasonic baths are easier to handle and more economical [53].

4.1.3.1 Ultrasonic bath

The extractions were conducted using an ultrasound water bath apparatus (Elmasonic S 40H, Elma Schmidbauer GmbH, Singen, Germany), set at a constant power of 340 W and a frequency of 37 kHz. 0,5 g of dried biomass of *Schizochytrium sp.* was weighed into 26 ml glass vials and 5 ml of NaDES was added in a ratio of 1:10. The vials were sealed with parafilm and set into the ultrasound bath with a custom made form. Before and after the extraction the temperature was measured using a handheld thermometer. The extraction temperature was varied from 30 to 60 °C according to the applied experimental design. After the extraction, the samples were quantitatively transferred to a falcon tube and centrifuged for 10 minutes at 6000 rpm (Digicen 21, Ortoalresa; Madrid, Spain). The supernatant was collected in a new falcon tube and stored in the refrigerator at 7°C until further analysis.

4.1.3.2 Ultrasonic probe

Extractions were carried out using an ultrasound probe system (UP200St; Sonotrode S26d2; Hielscher Ultrasonics, Teltow, Germany). The biomass of *Schizochytrium sp.* was weighed into 15 ml falcon tubes and the NaDES was added at a ratio of 1:10 (w/v). The falcon tubes were placed in an icebath to prevent overheating and degradation of the lipids during the extraction. The temperature was monitored throughout the process using a temperature probe, provided with the ultrasonic system to ensure consistent cooling. The extraction was conducted with the adjustment of the parameter's amplitude, pulse and time according to the applied experimental design with the probe immersion depth set to half of the medium's volume, approx. 2 cm. (Anmerkung [63])

4.1.4 Experimental design of ultrasound-assisted extraction using an ultrasonic bath

To identify the optimal extraction conditions, a response surface methodology (RSM) based on a Box–Behnken design (BBD) was employed. Separate second-order BBDs were developed for the ultrasonic bath and probe systems, each using three levels per variable and three replicates at the center point. The designs were generated with coded levels (−1, 0, +1), resulting in a total of 15 randomized experimental runs per system. This approach enables the modeling of linear, quadratic, and interaction effects while requiring fewer experiments than a full factorial design.

For the ultrasonic bath, using Me:Th as the NaDES system, the independent variables were extraction time (10, 20, 30 min), temperature (30, 45, 60 °C), and the molar ratio of the NaDES components (1:2, 1:1, 2:1). The complete design matrix and corresponding factor levels are summarized in Appendix Table A 1.

4.1.5 Experimental design of ultrasound-assisted extraction using an ultrasonic probe

A second Box–Behnken design was applied to optimize extraction using the ultrasonic probe. In this design, the molar ratio previously identified as optimal for the NaDES system was fixed, while three factors, time (2, 6, 10 min.), amplitude (40, 65, 90 %) and pulse (40, 70, 100 %), were varied at three levels.

A total of 15 randomized experimental runs were generated, again including three center-point replicates for estimation of pure error. Extractions were carried out in triplicate under the specific conditions defined by the design. The complete design matrix and corresponding factor levels are summarized in Appendix Table A 2.

For both models, analysis of variance (ANOVA) was used to assess model significance, individual factor effects, and model adequacy (including p-values, lack-of-fit, and R^2 values). Optimization was carried out using a desirability function to identify the most favorable combination of conditions for maximizing fatty acid yield. To validate the models, experimental extractions under the predicted optimal conditions were performed in triplicate. All designs were created and evaluated using OriginPro (OriginLab Corporation, Northampton, MA, USA). The response variable was the total fatty acid content, expressed as mg FA/g extract.

4.1.6 Reference extraction using conventional organic solvents

To evaluate the extraction performance of the NaDES systems in a meaningful context, reference extractions using conventional organic solvents were carried out. These methods serve as benchmarks, allowing for a direct comparison of extraction efficiency and providing a basis for the sustainability classification of the NaDES-based approaches relative to conventional solvent systems.

4.1.6.1 Soxhlet extraction using n-hexane

A conventional Soxhlet extraction using n-hexane was performed as reference method. For each replicate, 4.00 g of *Schizochytrium sp.* biomass were placed in a paper filter and extracted with 300 mL of n-hexane over a continuous period of 8 hours. Under typical operating conditions, the apparatus performs approximately 4–6 solvent recycling cycles per hour, corresponding to an estimated 32–48 cycles over the full extraction period. The extractions were carried out in triplicate. Upon completion, the hexane was evaporated using nitrogen until constant weight was reached and the total lipid yield was determined gravimetrically.

4.1.6.2 Ultrasound-assisted extraction using n-hexane

In addition, a second reference method was performed to directly compare the extraction efficiency of NaDES and conventional solvents under identical UAE conditions. For this purpose, *n*-hexane was used instead of NaDES, applying the optimal extraction parameters previously determined by RSM for both ultrasonic bath and probe. The solvent-to-biomass ratio was kept identical as in the corresponding NaDES experiments. After extraction, the hexane phase was transferred into a pre-weighed glass vial and the hexane was evaporated and the yield determined gravimetrically.

4.1.7 Sustainability assessment of the extraction methods

The sustainability of the sample preparation protocols was evaluated using the AGREEprep tool [64], which is based on the ten principles of Green Sample Preparation. Each method was scored from 0 to 1 across ten criteria, including sample amount, solvent and reagent use, energy consumption, number of steps, degree of automation, waste generation, and safety.

Input values were based on the experimental setup of the extraction and analysis methods developed in this work. Energy consumption was determined using an electricity usage monitor (Baldr, Model B3091-2), and reagent quantities were taken from laboratory records. Hazard and sustainability profiles of solvents and reagents were assessed using available literature and safety data sheets. The tool calculated an overall greenness score for each method, and individual criteria were analyzed to identify strengths and weaknesses.

The comparison included conventional Soxhlet extraction and the ultrasound-assisted extractions performed with both hexane and NaDES. For the ultrasonic bath and probe, only the RSM-derived optimal

conditions for each solvent system were used for the final comparison, and all extractions were assessed under identical AGREEprep criteria.

4.1.8 *Lipid Recovery after menthol:thymol extraction*

Various methods were applied to recover the extracted lipids from the NaDES phase, as the hydrophobicity, high viscosity and low volatility of these solvents pose a challenge for conventional separation techniques. To test the following techniques a model NaDES-oil mixture was prepared based on the expected lipid yield after extraction determined in a UAE with hexane under the same conditions as the NaDES (30 min./50°C) resulting in a yield of approximately 35 %. Prior to the recovery experiments, a solubility test had been conducted to confirm the miscibility of the oil in the selected NaDES system.

4.1.8.1 Physical separation approaches

Centrifugation was applied to exploit possible density differences between the lipids and the NaDES, aiming to induce phase separation under high centrifugal force.

Additionally, temperature-based methods were investigated, including freezing-induced and cold-induced separation, where the samples were stored overnight at -20°C and 7°C. These approaches were based on the assumption that lipid solubility in NaDES decreases at lower temperatures, which can lead to lipid aggregation or phase separation.

4.1.8.2 Antisolvents

The basic idea is that adding an antisolvent changes the polarity of the NaDES and thus reduces its ability to dissolve lipids. As a result, the NaDES becomes miscible with the antisolvent, while the less polar lipids tend to separate into a separate phase. This approach follows the method described by Lo et al. [39], who successfully added antisolvent to a semi-hydrophobic NaDES system based on imidazole and hexanoic acid, resulting in effective lipid separation.

To investigate the potential of antisolvents to enhance the separation of lipids from the NaDES phase, different solvents were tested: ethyl acetate, acetonitrile, acetone, 2-methyltetrahydrofuran (2-MeTHF), and tert-amyl methyl ether (TAME). For each test, 2.5 mL of the respective antisolvent was added to a separate Falcon tube containing the NaDES-oil mixture. Initial observations showed that all

antisolvents remained primarily on top of the NaDES-oil layer; however, upon vortexing and centrifugation (6000 rpm, 10 min.), it resulted in a single mixed phase, indicating no phase separation.

To further promote separation, an additional 2.5 mL of water was added to each system. After vortexing and centrifugation, a clear lower aqueous phase formed, while an upper phase consisting of NaDES, oil, and antisolvent remained, still without separation of oil from the NaDES.

A final experiment was conducted using Tween 80 as a demulsifier, based on its reported ability to disrupt NaDES-oil emulsions [65]. A concentration of 1 % Tween 80 was added to each of the Falcon tubes (with NaDES, oil, antisolvent, and water), as well as to two control tubes: one containing only NaDES and oil, and one containing NaDES, oil, and water. In the presence of water, Tween 80 led to the formation of emulsions but did not facilitate phase separation between NaDES and oil. In the system without water, Tween 80 was fully miscible with the NaDES-oil mixture, again resulting in a homogeneous phase with no visible separation.

While previous studies have shown that polar antisolvents such as water, methanol, and ethanol can induce phase separation in semi-hydrophobic DES systems for lipid recovery, experiments with a fully hydrophobic NADES did not yield similar results.

4.1.8.3 Liquid-liquid extraction

A different approach was to attempt a liquid-liquid extraction using conventional solvents to separate the lipids from the NaDES phase. However, it was found that solvents such as hexane are fully miscible with the hydrophobic NaDES, resulting in a homogeneous phase rather than a biphasic system. As a consequence, no phase separation occurred, and lipid extraction via this method was not feasible under the tested conditions.

4.1.9 GC–MS analysis of fatty acid methyl esters (FAMES)

The analysis of fatty acids was performed using GC-MS system (Agilent 7890B, Agilent Technologies, Santa Clara, CA, USA) . Prior to analysis, the samples were derivatized following a base-catalyzed transesterification protocol based on Castejón and Señoráns [66], with further adaptations. Initially, 25 mg of lipid extract were dissolved in 200 μ L of n-hexane (or standard solution), followed by the addition of 50 μ L of freshly prepared 2 M KOH in methanol. After vortexing for 1 minute and incubation at room temperature for 5 minutes, approximately 125 mg of sodium bisulfite ($\text{NaHSO}_4 \cdot \text{H}_2\text{O}$) were added to

neutralize the reaction. The mixture was vortexed again and centrifuged (5 min at 50 rpm). For analysis, 30 μL of the resulting supernatant were diluted with 1170 μL hexane (1:40 dilution). For quantification, methyl tricosanoate (C23:0) in hexane was used as an internal standard, which was freshly prepared each day to minimize errors caused by hexane evaporation and ensure consistent quantification conditions.

4.1.10 Calculation of fatty acid concentrations

To enable comparison between different extraction methods, the fatty acid content was expressed as milligrams of fatty acids per gram of extract (mg FA/extract). For each fatty acid (FA), the peak area was related to the area of the internal standard, and the concentration in mg mL^{-1} was calculated based on the known amount of internal standard injected. In cases where injection variability affected the reproducibility of the IS signal, the average IS peak area across the dataset was used for normalization to improve consistency.

$$\text{FA concentration (mg/mL)} = \left(\frac{\text{Area}_{\text{FA}}}{\text{Area}_{\text{IS}}} \right) \times c_{\text{IS}}$$

Formula 1: FA concentration (mg mL^{-1}) with Area_{FA} = peak area of the fatty acid; Area_{IS} = peak area of the internal standard; c_{IS} = concentration of the internal standard (3 mg mL^{-1}).

The resulting concentration was then multiplied by the dilution factor (DF = 40) to obtain the actual concentration in the original, undiluted extract solution:

$$\text{FA concentration in original solution} = \text{FA}_{\text{mg mL}^{-1}} \times 40$$

Formula 2: Calculation of dilution

This concentration was further related to the extract weight and the volume used in the analysis in order to calculate the amount of fatty acids per 25 mg of extract

$$\text{mg FA/25 mg extract} = \frac{\text{FA concentration in original solution} \times 0,2 \text{ ml}}{\text{extract weigh-in (mg)}}$$

Formula 3: Calculation of mg FA/25 mg extract

Finally, the result was scaled up to 1 gram of extract to provide the final value in mg FA/g extract, which served as the standardized basis for comparing extraction yields across all conditions.

$$\text{mg FA/g extract} = \text{mg FA/25 mg extract} \times 1000$$

Formula 4: Calculation of mg FA/g biomass

This standardized approach ensured accurate quantification and enabled reliable comparison of fatty acid yields across different extraction methods and conditions.

4.2 Fatty-acid-based NaDES

To expand the scope of this work, selected NaDES composed of medium-chain fatty acids and phenolic hydrogen bond donors were investigated. These systems were chosen based on literature reports highlighting their potential for reversible lipid extraction. The preparation and application of these NaDES followed the experimental strategy used in previous sections, with specific adjustments described below.

4.2.1 Preparation and characterization of fatty-acid-based NaDES

Based on literature, the C8:C12 system was prepared in a molar ratio of 3:1, as this composition has been reported to exhibit reversible phase behavior favorable for lipid release. For all other combinations (C8 or C12 with menthol, thymol, or carvacrol), an initial molar ratio of 1:1 was tested. However, systems containing C12 with menthol or thymol proved unstable under these conditions and were excluded from further experiments. Consequently, all remaining combinations were prepared at a molar ratio of 3:1 (fatty acid:HBD), resulting in the following NaDES systems: C8:Menthol (3:1), C8:Thymol (3:1), and C8:Carvacrol (3:1).

4.2.2 Application of optimized UAE conditions to fatty-acid-based NaDES

The UAE conditions previously optimized by response surface methodology were applied without modification to all fatty acid based NaDES systems. Based on the RSM results, only the more effective method, either ultrasonic bath or probe, was used for each case.

4.2.3 Lipid recovery using switchable hydrophobicity (Jeffamine method)

For Nades consisting of at least one fatty acids it was possible to utilize the switchable hydrophobicity of the fatty acid compounds to separate the Nades from the extracted lipids.

To recover the extracted lipids from NaDES consisting of at least one fatty acid, a strategy based on the reversible shift of hydrophobicity using a Jeffamine D-230 aqueous solution was applied, following the concept described by Sed et al. [44]. This method relies on the reversible formation of a hydrophilic phase through protonation/deprotonation, enabling phase separation between lipids and NaDES.

The method was initially tested using a NaDES composed of octanoic acid and dodecanoic acid in a molar ratio of 3:1. A 5% (v/v) Jeffamine D-230 solution was freshly prepared by dilution with distilled water. In the first trial, 5 mL of NaDES were combined with 65 mL of the Jeffamine solution, resulting in a 1:13 (v/v) ratio. This led to a complete polarity inversion of the NaDES, forming a single homogeneous hydrophilic phase.

In a second step, 2 mL of commercial *Schizochytrium* oil were added to the NaDES prior to the addition of the Jeffamine solution. After vortexing, the mixture was combined with 65 mL of the 5% Jeffamine solution. Upon mixing, the Jeffamine and NaDES formed a single hydrophilic phase, while the oil separated as a distinct hydrophobic phase on top. To determine the lipid recovery, the upper oil layer was collected using a Pasteur pipette and transferred into a pre-weighed vial. The yield was then calculated gravimetrically, taking into account the initial amount of oil added.

In a third step, the procedure was performed under real conditions following the ultrasound-assisted extraction using the ultrasonic probe. After extraction, the NaDES-extract mixture was transferred to a separatory funnel, where the Jeffamine solution was added. The funnel was gently shaken and then left undisturbed for 10 minutes to allow for phase separation. Subsequently, the lower Jeffamine-NaDES phase was drained into Falcon tubes, while the upper oil-rich phase was carefully collected using a Pasteur pipette and transferred into a separate 15 mL Falcon tube. All tubes were centrifuged at 6000 rpm for 10 minutes. Lipid fractions that had accumulated on top of the Jeffamine tubes were transferred into the oil tube, which was centrifuged again under the same conditions. The final upper phase was transferred into a pre-weighed vial, and the lipid yield was determined by gravimetric difference.

Since preliminary tests showed that the extraction yield for some NaDES combinations frequently exceeded 100 %, an additional step was introduced to reduce the apparent extract mass. For this purpose, the same Jeffamine-based phase separation protocol was applied a second time, using only the previously recovered extract while maintaining the same Jeffamine ratio.

The main objective of the procedure was the recovery of the lipid-rich extract from the NaDES phase. In addition, the potential to separate the NaDES itself from the Jeffamine solution via reversible polarity switching was evaluated. To induce rehydrophobization and allow phase separation, hydrochloric acid (2 N, 37%) was added dropwise to the hydrophilic Jeffamine-NaDES mixture until a visible biphasic system was formed. This confirmed that the NaDES could be returned to a hydrophobic state and separated from the aqueous phase.

4.2.4 *Analysis of extracts obtained with fatty-acid-based NaDES*

To comprehensively evaluate the composition and quality of the lipid extracts obtained with fatty-acid-based NaDES, a combination of analytical approaches was applied. First, the overall lipid yield was determined gravimetrically by difference weighing and related to the initial biomass input. Additionally, conventional GC-MS analysis was used to quantify the fatty acid content in mg FA per g extract. To assess the presence of NaDES residues in the extract, a targeted acid-catalyzed derivatization method was implemented. This enabled the detection of free fatty acid components originating from the NaDES itself, allowing a more accurate interpretation of extract purity and recovery.

4.2.4.1 Acid derivatization for extract composition analysis

This method was adapted from Christie [67] and chosen specifically because standard base-catalyzed methylation failed to reliably detect short-chain free fatty acids in the chromatogram. Approximately 10 mg of dried extract were dissolved in 1 mL of 1 % sulfuric acid in methanol (v/v) and incubated at 35 °C for 30 minutes under constant shaking to ensure complete esterification. Following derivatization, 2 mL of hexane were added to extract the resulting FAMES. The organic phase was washed with 3 mL of 2 % sodium bicarbonate (w/v), and the upper layer was subsequently passed through a small filter containing anhydrous sodium sulfate (Na_2SO_4). The filter was then rinsed with 1 mL of hexane. After evaporation to constant weight, the FAMES were redissolved in 600 μL hexane and analyzed via GC-MS.

4.3 Cell viability testing

To evaluate the potential cytotoxic effects of the NaDES components and the corresponding lipid extracts, two complementary assays (CellTiter-Blue® and Sulforhodamine B) were conducted using HCEC-

1CT and HCT116 cells. The following sections describe the cultivation, treatment, and assay procedures in detail.

4.3.1 *Materials, reagents and cell lines*

Fetal calf serum (FCS), 1 M HEPES buffer solution, gentamicin solution, 10× Medium 199, and 100× insulin-transferrin-selenium-G (ITS) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant human epidermal growth factor (EGF) and HyClone™ Cosmic Calf™ serum were purchased from Corning® Inc. (Corning, NY, USA) and GE Healthcare Life Sciences (Piscataway, NJ, USA), respectively. Hydrocortisone and Accutase® were purchased from Sigma-Aldrich (Schnelldorf, Germany). Cell culture disposables were obtained from Sarstedt AG + Co. KG (Nuembrecht, Germany) and Carl Roth GmbH + Co. KG (Karlsruhe, Germany). The non-tumorigenic human colonic epithelial cell line HCEC-1CT, kindly provided by Prof. Jerry W. Shay (UT Southwestern Medical Center, Dallas, TX, USA), and the human colorectal carcinoma cell line HCT116 (ATCC, Manassas, VA, USA) were used in this study.

For comparative purposes, the human colorectal carcinoma cell line HCT116 (ATCC, Manassas, VA, USA) was cultivated in DMEM GlutaMAX™ (Thermo Fisher Scientific) supplemented with 10 % FCS and 1 % penicillin/streptomycin (P/S). All cultures were maintained under sterile conditions at 37 °C in a humidified atmosphere containing 5 % CO₂.

4.3.2 *Cell culture and maintenance*

HCEC-1CT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose; Thermo Fisher Scientific, Vienna, AUT) supplemented with 2 % HyClone™ Cosmic Calf™ serum, 2 % 10× Medium 199, 20 mM HEPES buffer, 50 µg mL⁻¹ gentamicin, 1 µg mL⁻¹ hydrocortisone, 10 µL/mL Insulin-Transferrin-Selenium-G supplement, and 20 ng/mL recombinant human EGF.

HCT116 cells were cultivated in DMEM GlutaMAX™ (Thermo Fisher Scientific) supplemented with 10 % FCS and 1 % penicillin/streptomycin (P/S).

Cell lines were maintained at 37 °C in a humidified atmosphere containing 5 % CO₂ and passaged twice weekly using Accutase® (HCEC-1CT) or Trypsin (HCT116) for detachment. All reagents were pre-warmed to 37 °C prior to use, and all procedures were performed under sterile biosafety level 2 (BSL-2) conditions.

4.3.3 Treatment preparation and concentration range

All cytotoxicity assays were performed using both HCEC-1CT and HCT116 cells under identical treatment conditions. In particular, cells were exposed to (i) lipid extracts obtained from ultrasound-assisted extraction with different NaDES compositions (C8:C12, C8:Th, C8:Me, C8:Ca, Me:Th), (ii) the pure NaDES solvent mixtures used for the extraction of lipids, (iii) the individual NaDES components (menthol, thymol, carvacrol, octanoic acid (C8) and lauric acid (C12)), and (iv) Jeffamine D-230, which was used for phase separation during lipid recovery.

For the lipids extracted using NaDES, the pure NaDES mixtures, the Soxhlet-extracted lipids, and a commercial *Schizochytrium sp.* oil reference, three concentrations (1, 10, and 100 $\mu\text{g mL}^{-1}$) were tested. These concentrations were selected to encompass a range from sub-cytotoxic to potentially cytotoxic levels while maintaining DMSO at a non-toxic concentrations of 0.25 % (v/v) in all wells.

The concentration ranges for the individual NaDES components were adjusted according to their approximate molar fraction within the corresponding NaDES mixtures to reflect realistic exposure levels. Accordingly, menthol and thymol were tested at 25, 75, and 100 $\mu\text{g mL}^{-1}$; carvacrol at 25 and 100 $\mu\text{g mL}^{-1}$; caprylic acid at 75 and 100 $\mu\text{g mL}^{-1}$; and lauric acid at 25 and 100 $\mu\text{g mL}^{-1}$. For Jeffamine D-230, the concentrations 1, 10, and 100 $\mu\text{g mL}^{-1}$ were chosen, as its residual amount in the recovered extracts could not be precisely quantified.

Samples were dissolved in DMSO (to prepare 400 $\times$ stock solutions) and diluted with cell culture medium immediately before cell treatment. Cells exposed to 0.25% DMSO served as negative control, while 0.1 % Triton X-100 was used as a positive cytotoxicity control.

4.3.4 CellTiter blue® (CTB) assay

Cell viability was determined using the CellTiter-Blue® (CTB) assay, which allows to assess the metabolic conversion of resazurin to the fluorescent product resorufin by viable cells. Both the non-tumorigenic colonic epithelial cell line HCEC-1CT and the colorectal carcinoma cell line HCT116 were used for assessment under identical treatment conditions. Cells were seeded in sterile 96-well plates (flat-bottom; yellow plates: Sarstedt's Cell+ growth surface) at a density of 5×10^3 cells per well in 100 μL of complete growth medium, while outer wells were filled with PBS to minimize edge effects. Following seeding, HCEC-1CT were pre-incubated for 24 h and HCT116 for 48 h to achieve comparable confluence prior to exposure. The medium was then replaced with 100 μL of the test media containing the NaDES

or lipid extracts described (see section 4.2.3), and cells were incubated for 24 h at 37 °C in a humidified 5 % CO₂ atmosphere. After treatment, the test media were carefully aspirated and replaced with CTB working solution prepared by diluting 1:10 of the CTB reagent with phenol-red-free DMEM. Plates were incubated for 1 h and the fluorescence signal was measured with a plate reader (λ_{ex} 560 nm, λ_{em} 590 nm). Blank wells containing only the CTB working solution served for background correction. Cell viability was expressed as percentage of fluorescence relative to the solvent control.

4.3.5 *Sulforhodamine B assay (SRB)*

To quantify total cellular protein content as an indirect measure of cell number, the sulforhodamine B (SRB) assay was performed. Following CTB fluorescence measurement, the same plates were immediately processed for SRB staining. At the end of treatment, cells were fixed by adding 10 μ L cold 50 % trichloroacetic acid (TCA) per well and incubated for 1 h at 4 °C in the dark. Plates were washed 4 times with tap water and air-dried overnight. Cells were stained with 50 μ L 0.4 % (w/v) SRB in 1 % acetic acid for 60 min at room temperature in the dark. Unbound dye was removed by washing twice with tap water and twice with 1 % acetic acid. After drying, bound dye was solubilized in 100 μ L 10 mM Tris base (pH 10), and absorbance was measured at 570 nm. Blank values, obtained from cell-free wells subjected to the SRB staining procedure, were subtracted from all samples.

4.3.6 *Data processing and statistical analysis*

All conditions were tested in triplicate within each experiment, and data are presented as mean $\pm$ standard deviation from at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA). For extraction-related data (e.g., optimization and yield comparison), significant differences between groups were evaluated using Tukey's post hoc test. For cytotoxicity assays, Dunnett's post hoc test was applied to identify significant differences compared to the untreated control, while Fisher's least significant difference (LSD) test was additionally used for exploratory pairwise comparisons among NaDES treatments. All analyses were conducted using OriginPro, and differences were considered statistically significant at $p < 0.05$.

5 Results and Discussion

5.1 Lipid extraction strategies using menthol- and thymol-based NaDES

5.1.1 Physicochemical characterization of menthol-thymol NaDES

For the extraction of lipids from *Schizochytrium sp.* using Me:Th, three molar ratios (1:2, 1:1, and 2:1) were prepared and examined. All mixtures formed clear, homogeneous liquids at room temperature without visible crystallization or phase separation, confirming the successful formation of a Type V eutectic system [34].

To quantify the density of the individual mixtures, measurements were performed at room temperature using a gravimetric pipette-tip method. The results summarized in Table 2 show a systematic decrease in density with increasing menthol content, ranging from 0.956 g mL⁻¹ (1:2) to 0.932 g mL⁻¹ (2:1). This trend aligns well with the densities of the pure components, with thymol exhibiting a higher density (~1.03 g mL⁻¹) than menthol (~0.89 g mL⁻¹).

Table 2: Density of menthol-thymol NaDES at different molar ratios

Molar ratio	Density (g/mL)
1:2	0.956 ± 0.001
1:1	0.945 ± 0.001
2:1	0.932 ± 0.003

Small deviations from individual literature values may be attributed to differences in temperature control and measurement technique. In this work, density was determined gravimetrically at ambient laboratory temperature, whereas literature data were obtained with high-precision vibrating-tube densimeters under tightly controlled thermal conditions. For example, reported densities for the menthol:thymol 1:1 system range between 0.93656 g mL⁻¹ and 0.93812 g mL⁻¹ (measured at 25 °C). A similar trend is observed for the 2:1 mixture, where literature reports 0.92384 g mL⁻¹ and 0.92556 g mL⁻¹ (25 °C) [32], [68]. Even a temperature difference of 2–4 °C can shift density values of NaDES by several 10⁻³ g mL⁻¹. Furthermore, the water contents reported for menthol-thymol NaDES typically range from 200 to 1100 ppm after preparation, which can introduce additional density variations in the same order

of magnitude [68]. Considering these factors, the observed differences are fully consistent with expected experimental variability.

To evaluate the temporal stability of the eutectic mixture and ensure that the mixing process had reached completion, density measurements were performed at multiple time points during preparation. No significant changes were observed between 15 minutes and 16 hours, indicating that the eutectic phase forms rapidly and remains structurally stable over time. Such behavior is consistent with previous observations that terpene-based NaDES rely primarily on strong but rapidly establishing hydrogen-bond interactions to achieve a stable low-melting mixture [69]

5.1.2 *Ultrasound-assisted extraction with bath system (RSM optimization)*

The extraction efficiency of the menthol-thymol NaDES was optimized using a Box–Behnken response surface design including three independent variables: extraction time (10–30 min), temperature (30–60 °C), and molar ratio (1:2, 1:1, 2:1). In total, 15 experimental runs were performed to evaluate linear, quadratic, and interaction effects. The following part focuses on the statistical model performance, the influence of individual factors, and the identification and validation of optimal extraction conditions. The statistical performance of the model was evaluated based on R^2 , adjusted R^2 , lack-of-fit analysis and the significance of individual terms. A complete overview of the regression coefficients and ANOVA results is provided in the Appendix (Table A 3 – Table A 5).

The resulting regression model achieved an R^2 of 0.5003 and an adjusted R^2 of 0.3719, which together provide an estimate of how well the experimental data are described by the fitted response surface. The R^2 value of 0.5003 means that the model explains roughly 50 % of the variation in fatty acid yield across the tested experimental conditions, meaning half of the changes observed in the extraction efficiency can be attributed to systematic effects of time, temperature, and molar ratio as captured by the regression equation, whereas the remaining variation is caused by factors not included in the model or by experimental noise. The adjusted R^2 (0.3719) provides a more conservative estimate that accounts for the number of fitted parameters. The lower adjusted R^2 indicates that some model terms contribute only marginally to improving prediction accuracy, which is expected in systems with moderate biological variability and weak curvature effects.

To evaluate whether the fitted surface adequately represents the underlying data, the lack-of-fit test was inspected. It compares how much the model's predictions deviate from the measured values with the amount of variation that naturally occurs between replicated experiments. Because the lack-of-fit

was not significant ($p = 0.1039$), the discrepancies between predicted and observed yields are consistent with normal experimental variability rather than systematic model error. In practical terms, this means that the model provides a sufficiently accurate description of how the three extraction parameters influence the fatty acid yield within the investigated range.

Among the linear factors, extraction time showed the only statistically significant effect on the fatty acid yield ($p = 0.005$). This means that changes in sonication time consistently produced measurable changes in the response that were larger than the natural experimental variability. Temperature displayed a positive trend ($p = 0.077$), indicating that higher temperatures tended to increase the yield, although the effect was not strong enough to be classified as statistically reliable within the tested range. The molar ratio showed no significant linear influence, meaning that variations between the 1:2, 1:1, and 2:1 combinations did not systematically shift the extraction outcome.

A statistically significant interaction was detected between extraction time and temperature ($p = 0.0019$). An interaction effect means that the influence of one factor depends on the level of another factor; here, the effect of temperature becomes more noticeable at longer extraction times. All other interactions were statistically non-significant, implying that combinations of factors other than time $\times$ temperature did not produce systematic changes in the response. A significant quadratic effect was found for the molar ratio ($p = 0.0056$), indicating a slight curvature in the response around the central 1:1 composition. In contrast, the quadratic terms for time and temperature did not contribute meaningfully, suggesting that within the investigated range the response changed mostly in a linear rather than curved fashion for these two variables.

To verify that the regression model meets the underlying statistical assumptions, the behavior of the residuals, the differences between the measured and predicted fatty acid yields, was examined (Figure 2). Ideally, residuals should appear as random noise without recognizable patterns, they represent the differences between the measured extraction yields and the values predicted by the regression model. In the plot against the experimental run order, the residuals showed no systematic drift, indicating that the measurements remained stable throughout the experiment. When plotted against the fitted values, the residuals were evenly scattered around zero, suggesting that the variability of the errors was consistent across the prediction range and that no missing non-linear trends remained. The histogram displayed an approximately symmetric distribution centered around zero, a single more pronounced negative outlier was visible, but it did not follow a systematic pattern and is therefore interpreted as normal experimental variability rather than a structural model deviation. The normal probability plot

aligned closely with the reference line, indicating that the residuals followed a roughly normal distribution. Overall, these diagnostic plots confirm that the model errors behave as expected for a valid regression.

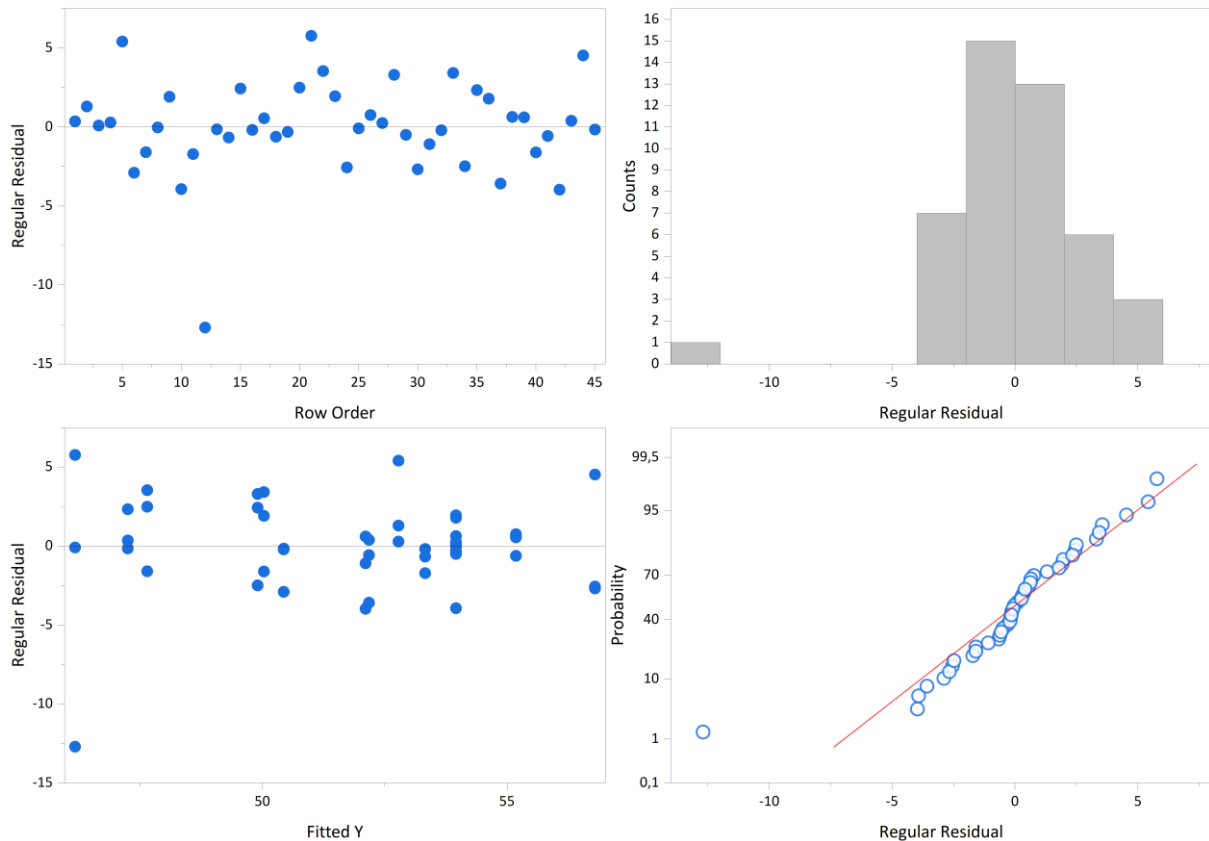


Figure 2: Residual diagnostic plots for the Box–Behnken response-surface model of Me:Th extraction using the ultrasonic bath.

Residuals versus row order (top left) and residuals versus fitted values (bottom left) assess independence and homoscedasticity of the model errors. The histogram of residuals (top right) and the normal probability plot (bottom right) evaluate their distributional properties.

The standardized effects plot (Figure 3) visualizes the relative magnitude of each model term and highlights which factors had the strongest influence on the fatty acid yield. The largest bar corresponds to the interaction between extraction time and temperature, confirming that this combined effect was the dominant contributor to changes in the response. Extraction time also showed a substantial individual effect, consistent with the observation that longer sonication generally increased extraction efficiency. A smaller but still notable bar was observed for the quadratic term of the molar ratio, indicating a slight curvature around the central composition level.

In contrast, temperature alone produced a moderate but weaker effect, and the linear term of the molar ratio contributed only minimally within the tested range. All remaining interaction and quadratic terms appear far below the significance threshold, illustrating that they had little impact on the response. Overall, the plot provides a visual summary of the model structure and confirms that the extraction behavior was primarily shaped by sonication time and its interaction with temperature, while variations in the Me:Th composition played a comparatively minor role.

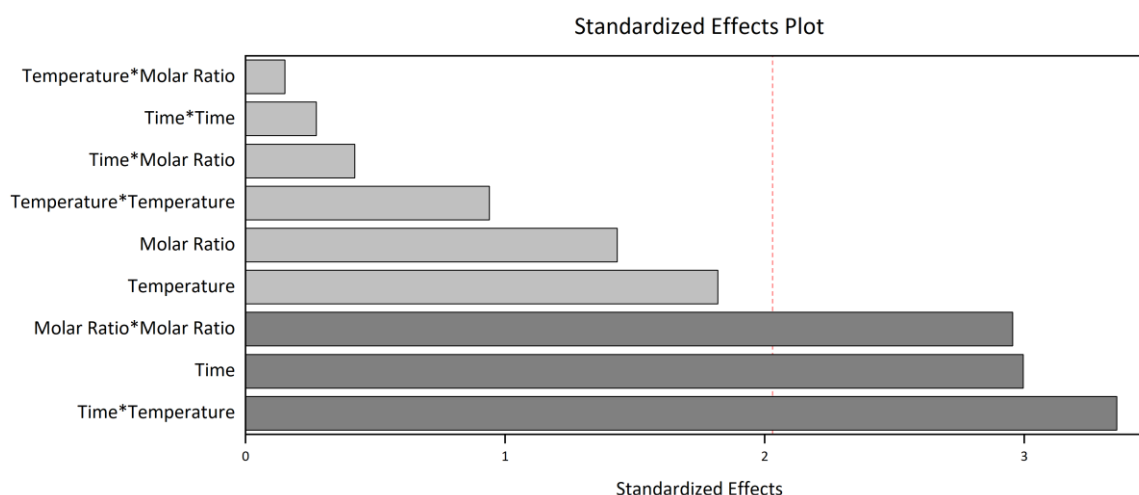


Figure 3: Standardized effects plot of the Box–Behnken response-surface model for Me:Th extraction using the ultrasonic bath.

Bars represent the standardized effects of all linear, interaction, and quadratic terms on the fatty acid yield. The vertical dashed line indicates the significance threshold ($\alpha = 0.05$). Dark bars denote significant effects, highlighting the dominant influence of extraction time and the interaction between time and temperature, while lighter bars represent non-significant factors.

The main effects plots (Figure 4) illustrate how each factor influences the fatty acid yield when considered individually. Extraction time showed a clear upward trend, with longer sonication steadily increasing the predicted yield, indicating that extended ultrasound exposure enhances the extraction process. Temperature displayed a curved profile: the predicted yield increased from 30 °C up to an optimum around 50–55 °C, after which the curve flattened or slightly declined. This suggests that moderate heating supports the extraction, whereas higher temperatures offer diminishing returns. The molar ratio exhibited a similar curved pattern, with the highest predicted yield near the central 1:1 composition and lower values at both more thymol-rich (1:2) and menthol-rich (2:1) ratios. This indicates that the solvent composition has an optimum balance point, beyond which deviations reduce extraction efficiency.

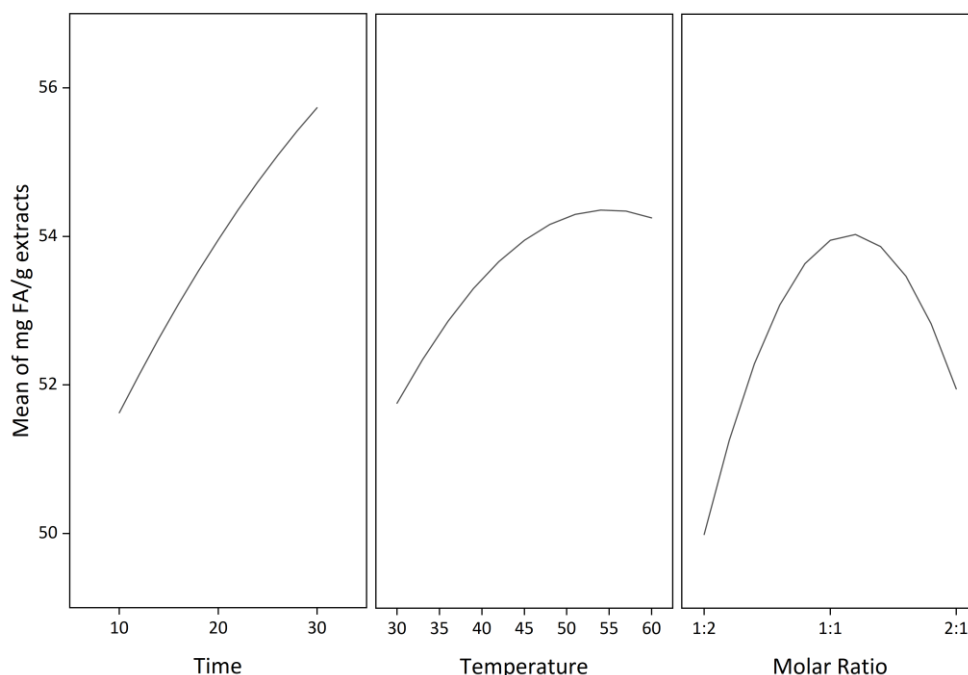


Figure 4: Main effects plots for extraction time, temperature, and molar ratio in the Box–Behnken model of Me:Th extraction using the ultrasonic bath.

The interaction plots (Figure 5) illustrate how pairs of factors jointly affect the extraction outcome and whether their combined influence differs from the sum of their individual effects. Each panel shows one factor on the x-axis and three lines representing the levels of the second factor. If the lines run roughly parallel, the two factors act independently; if the lines diverge or converge, the strength of one factor’s effect changes depending on the level of the other. When lines cross, this indicates a strong interaction, meaning that the direction or magnitude of the effect of one factor reverses depending on the second factor’s setting. The most pronounced interaction was observed between extraction time and temperature: at lower temperatures, increasing time produced only modest changes in the predicted yield, whereas at higher temperatures, the same increase in time resulted in substantially larger improvements. This divergence in slope indicates that the effect of time becomes stronger when the extraction is carried out at elevated temperatures.

Interactions involving the molar ratio showed only subtle differences between the lines, reflecting the small contribution of this factor to the overall model. While slight curvature is visible in the molar ratio profiles, consistent with the quadratic effect observed earlier, the distance between the curves remains narrow, indicating that changes in solvent composition only minimally modify the influence of time or temperature. Interactions among quadratic terms and other combinations displayed nearly parallel lines, suggesting largely additive behavior without meaningful synergistic or antagonistic

effects. Overall, the interaction plots confirm that the only relevant dependence between factors occurs between time and temperature, while all other combinations contribute little to the extraction behavior.

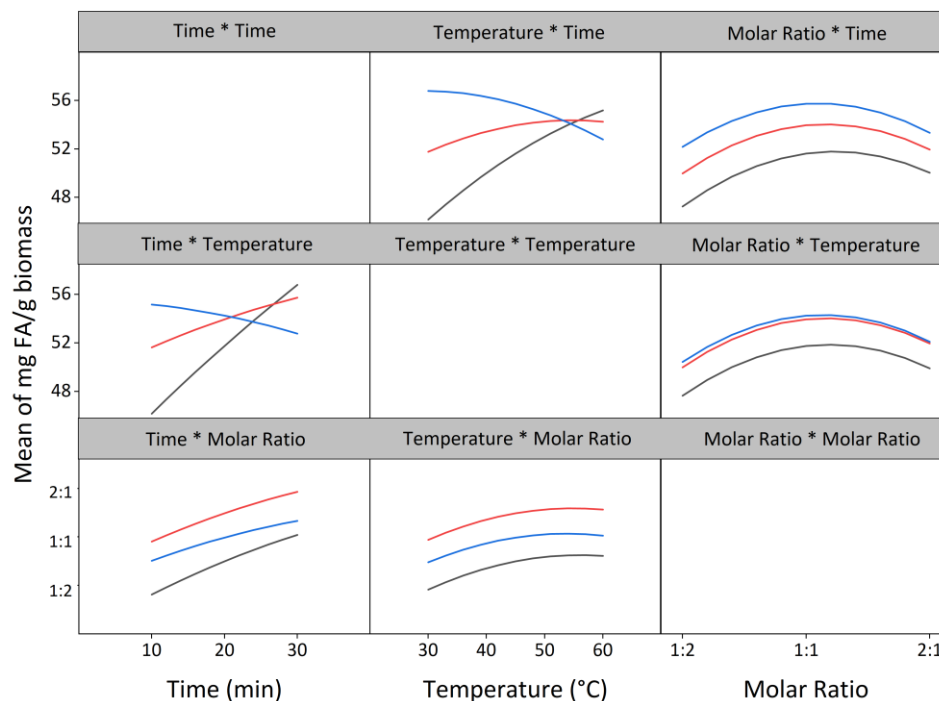


Figure 5: Two-factor interaction plots for the Box–Behnken response-surface model of Me:Th extraction using the ultrasonic bath.

In each panel, the x-axis represents the primary factor while the three colored lines depict the predicted response at the low (black), center (red), and high (blue) level of the second factor involved in the interaction.

Before analyzing the contour and surface plots, it is important to note that they are generated by holding the third factor at the optimal level predicted by the RSM model. The optimization identified an extraction time of 30 min, a temperature of 30 °C, and a 1:1 molar ratio as the best-performing conditions. This allows the plots to clearly show how the two changing factors work together near the predicted optimum and how they influence the yield.

The contour plots (Figure 6) provide a two-dimensional representation of how pairs of factors interact to shape the extraction performance. Across the three contour plots, the absolute yield ranges differ depending on which factor is held constant. With fixed time, predicted yields span 49.36–56.82 mg FA/g extract; with fixed temperature, the range broadens to 41.65–56.85 mg FA/g extract; and with fixed molar ratio, values fall between 46.15 and 56.80 mg FA/g extract. These differences in minimum

yields show that low temperatures have the strongest suppressive effect on performance, whereas variations in time and molar ratio mainly influence the upper part of the response surface.

The temperature-molar ratio plot shows that the highest predicted yields occur at lower to moderate temperatures (approximately 30–40 °C) and around the 1:1 to 2:1 combinations. Toward higher temperatures (≥ 50 °C), the extraction efficiency decreases noticeably, especially for the molar ratios 1:2 and 2:1 while staying more constant at a ratio of 1:1. The time-temperature plot shows a diagonal zone of high yields, with one maximum at high temperature and short extraction time and another at low temperature and long extraction time. Both factors positively influence the extraction, but the highest responses arise when one factor is at its upper level while the other remains moderate. When both time and temperature are simultaneously high, the predicted yield decreases slightly, indicating that the factors do not act synergistically. The time–molar ratio plot displays curved contours, indicating that molar ratio modifies the extraction less strongly than extraction time. Peak yields occur around an approximately equimolar composition ($\approx 1:1$) over the whole time range, while both more thymol-rich (1:2) and more menthol-rich (2:1) mixtures give slightly lower predicted values. Overall, the contour plots highlight that optimal extraction conditions emerge from a combination of low to moderate temperatures, extended sonication times, and balanced solvent composition.

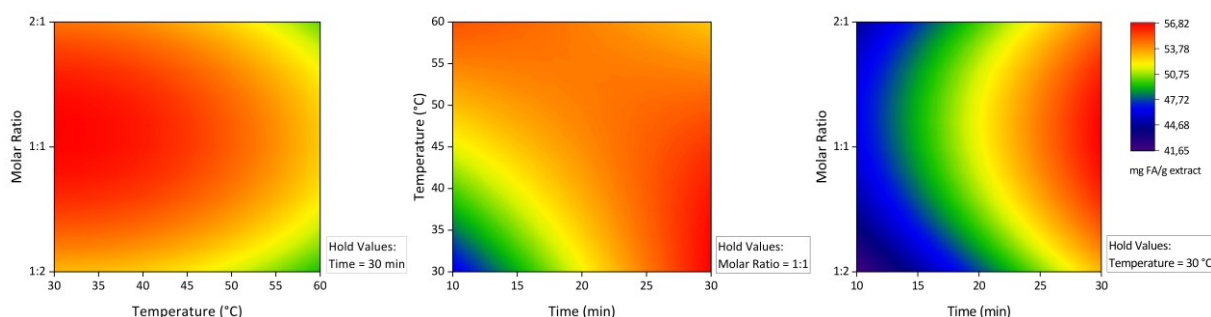


Figure 6: Contour plots of the Box–Behnken response–surface model for Me:Th extraction using the ultrasonic bath.

(a) Interaction between temperature and molar ratio (time held constant at 30 min); (b) Interaction between temperature and time (molar ratio held at 1:1); (c) Interaction between molar ratio and time (temperature held at 30 °C).

The 3D response surfaces (Figure 7) provide additional insight by revealing the curvature and overall shape of the extraction landscape more clearly than the contour plots alone. The temperature–molar ratio plot shows a broad, dome-shaped region of high yields, indicating that moderate temperatures and near-equimolar compositions create a wide plateau of favorable conditions. The yield is decreasing

gradually toward both more extreme temperatures and more asymmetric solvent ratios. This indicates that the extraction response is relatively stable within a moderate operating window but becomes less favorable when either factor deviates too far from its central level.

The time–temperature plot shows that the two maxima form a continuous elevated ridge, with the 3D surface clearly revealing its curved, saddle-like shape and the gentle decline toward the corners where both factors are either at their lowest or highest. The time–molar ratio plot shows a smoothly rising surface toward longer extraction times. It also highlights the gentle curvature along the molar ratio axis, confirming that changes in composition shift the surface only slightly compared with the strong, steady lift produced by increasing time. Together, these surfaces highlight that extraction time and temperature dominate the response landscape, whereas variations in solvent composition have a subtler, secondary influence.

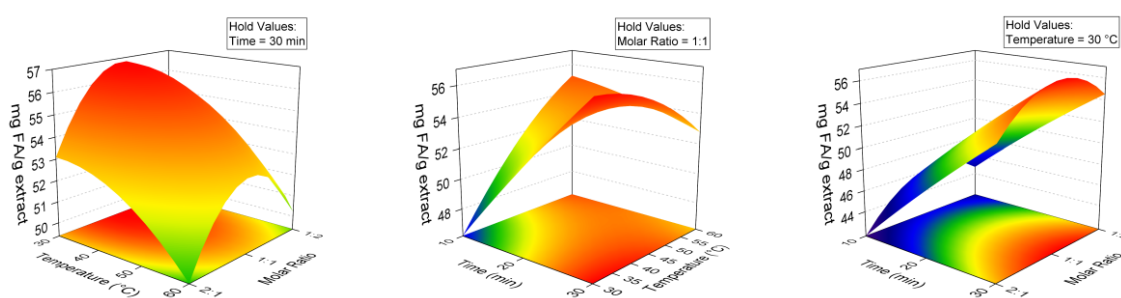


Figure 7: Three-dimensional response surface plots for the Box–Behnken model of Me:Th extraction using the ultrasonic bath.

(a) Interaction between temperature and molar ratio (time held constant at 30 min); (b) Interaction between temperature and time (molar ratio held at 1:1); (c) Interaction between molar ratio and time (temperature held at 30 °C).

Based on the full dataset of 45 extractions, the response–surface model was used to identify the parameter combination that provides the highest fatty acid yield. The model predicted that optimal performance would be reached at an extraction time of 30 minutes, a temperature of 30 °C, and a molar ratio corresponding to a menthol-to-thymol ratio of approximately 1.04 to 1, which is essentially equivalent to a practical 1:1 composition and was therefore rounded to an exact 1:1 ratio for practical implementation in the extraction experiment. These conditions were validated experimentally in three independent extractions. Under these conditions, a predicted yield of 56.84 mg FA/g extract was calculated with a composite desirability of 0.839 indicating that the model considered these conditions highly favorable for achieving the optimization target. Experimental validation under these settings

resulted in a mean extraction yield of 55.85 ± 0.44 mg/g extract ($n = 3$). To ensure consistent quantification, the internal standard signal was normalized using the average peak area obtained from 45 preceding GC–MS runs.

The optimization of Me:Th-based lipid extraction in the ultrasonic bath showed that the extraction behavior is mainly driven by time- and temperature-dependent mass-transfer processes. Longer sonication and moderate heating enhanced cavitation efficiency, improved solvent penetration, and accelerated lipid diffusion from the disrupted cell matrix. The strong time $\times$ temperature interaction reflects this synergistic effect, confirming that thermal support amplifies the mechanical impact of ultrasound. In comparison, the Me:Th molar ratio played only a secondary role. The limited variation in polarity and viscosity across the tested compositions explains why changes in solvent ratio produced only subtle effects. The slight optimum near the 1:1 composition indicates a balanced hydrogen bonding structure but contributes far less to the extraction outcome than the process parameters.

Nevertheless, several limitations should be acknowledged. The moderate adjusted R^2 indicates that additional factors not included in the model, such as particle-size distribution, biomass hydration state, or solvent–cell interactions, may also influence extraction efficiency. Moreover, the variability in internal standard responses highlights that analytical uncertainty can subtly affect absolute yield estimates, although this was effectively mitigated through averaging strategies. Finally, bath sonication inherently provides weaker cavitation intensities than probe-based systems, which may restrict the achievable extraction rates and could explain why time remains the dominant factor.

Despite these constraints, the results clearly demonstrate that Me:Th performs robustly under bath-assisted extraction and benefits substantially from longer sonication times combined with moderate heating. The method thus provides an accessible and reproducible extraction strategy with well-defined optimal parameters and predictable behavior across the tested range.

5.1.3 *Ultrasound-assisted extraction with probe system (RSM optimization)*

The extraction efficiency of the Me:Th NaDES was further optimized using a Box–Behnken design applied to the probe-based ultrasound system. Three process variables were investigated: extraction time (2–10 min), amplitude (60–90 %) and pulse (40–100 %) as independent variables. A total of 15 experimental runs were performed to evaluate linear, quadratic, and interaction effects, following the same design structure used for the ultrasonic bath. The behavior of the system was assessed based on the model fit, the significance of individual terms, and the overall predictive performance. A complete

overview of the regression coefficients and ANOVA results is provided in the Appendix (Table A 8 – Table A 10).

The probe-based model exhibited a markedly stronger explanatory power than the bath-assisted system, reaching an R^2 of 0.8566 and an adjusted R^2 of 0.8312. These values indicate that more than 80 % of the variation in fatty acid yield is captured by the fitted surface, reflecting the higher extraction efficiency and stronger process control typically associated with probe sonication. The predicted R^2 of 0.7808 shows good agreement with the adjusted R^2 , confirming that the model possesses solid predictive ability without signs of overfitting.

The lack-of-fit test was slightly significant with a p-value of 0.045, which means that the model does not match the experimental values perfectly. However, this small deviation is not critical. Probe sonication produces much stronger and less uniform cavitation than the ultrasonic bath, which naturally increases the variability of the extraction values. This explains why the model shows a minor lack-of-fit, even though the overall fit is excellent. The important point is that most of the unexplained variation comes from pure error, which was 982.3, while the lack-of-fit component was only 277.1. This indicates that the variability is largely caused by the natural differences observed between the replicate measurements rather than by shortcomings of the model itself. Together with the high R^2 of 0.8566 and adjusted R^2 of 0.8312, the model can still be considered reliable, and it is well suited to interpret the effects of the extraction parameters and to identify optimal conditions.

The ANOVA results show that all three linear factors, time, amplitude, and pulse mode, had a significant and positive influence on the extraction yield. Among them, amplitude was the dominant variable, reflecting the central role of acoustic power in probe sonication, where higher amplitudes generate stronger and more frequent cavitation events. Time and pulse mode also contributed significantly, indicating that both prolonged energy input and the temporal pattern of sonication pulses influence lipid release.

The quadratic terms revealed additional curvature in the response: amplitude showed a significant second-order effect, suggesting the presence of an optimum at intermediate intensity levels rather than a simple monotonic increase. A weaker but still relevant quadratic effect was observed for extraction time, whereas the pulse mode displayed no notable curvature within the tested range. Interaction effects between the factors were not significant, meaning that the influence of each variable remained largely independent and did not systematically change across the levels of the others.

Overall, the significant linear and quadratic terms explain the high predictive performance of the model and reflect the strong sensitivity of probe-based extraction to both the strength and duration of ultrasonic energy input.

To verify that the regression model for the probe-assisted extraction fulfils the required statistical assumptions, the residual diagnostic plots (Figure 8) were examined. The residuals showed no recognizable structure and appeared as random variation, indicating that the errors behaved as expected. When plotted against the experimental run order, no drift or grouping appeared, indicating that the measurements were stable throughout the experiment. The residuals versus fitted values were also evenly scattered around zero, which shows that the error variability was consistent and that no missing non-linear trends remained. The histogram displayed a roughly symmetric distribution centered near zero, with a slightly wider spread than in the bath system. The normal probability plot followed the reference line closely, apart from a few deviations at the extremes. Overall, the diagnostic plots confirm that the model residuals behave as expected and that the regression model provides a reliable description of the probe extraction data.

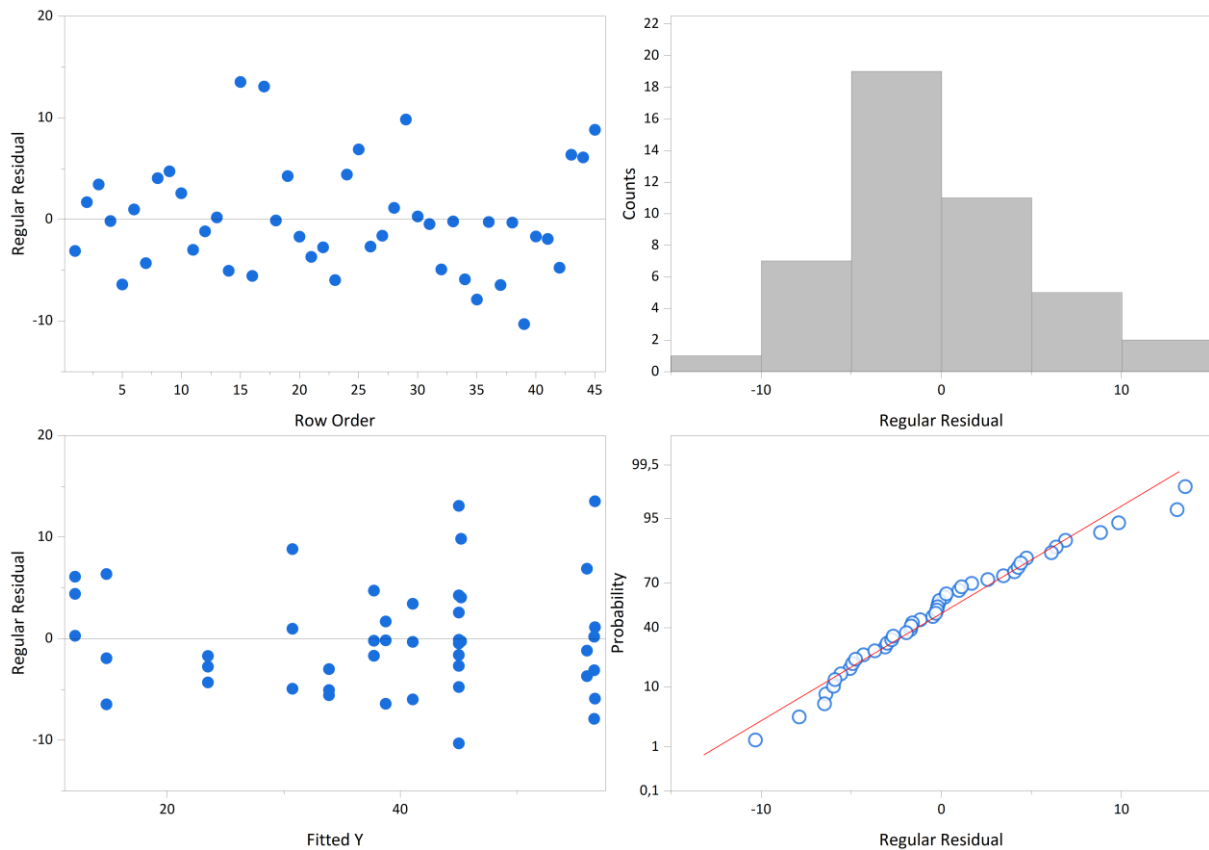


Figure 8: Residual diagnostic plots for the Box–Behnken response-surface model of Me:Th extraction using the ultrasonic probe.

Residuals versus row order (top left) and residuals versus fitted values (bottom left) assess independence and homoscedasticity of the model errors. The histogram of residuals (top right) and the normal probability plot (bottom right) evaluate their distributional properties.

The largest bar in the standardized effects plot (Figure 9) corresponds to amplitude, indicating that this parameter had the strongest overall impact on the response. Time and pulse also showed substantial individual effects, confirming that all three process variables contributed meaningfully to extraction performance. A notable quadratic effect was observed for amplitude, while the quadratic term of time was smaller but still above the significance threshold.

In contrast, the quadratic effect of pulse and the interaction terms involving time, amplitude, and pulse were all positioned well below the significance line, demonstrating that these combinations had little influence on the extraction outcome. Overall, the plot provides a clear summary of the model structure and shows that the behavior of the probe-based extraction was dominated by the direct effects of amplitude, time, and pulse, whereas interactive effects between these factors played only a minor role.

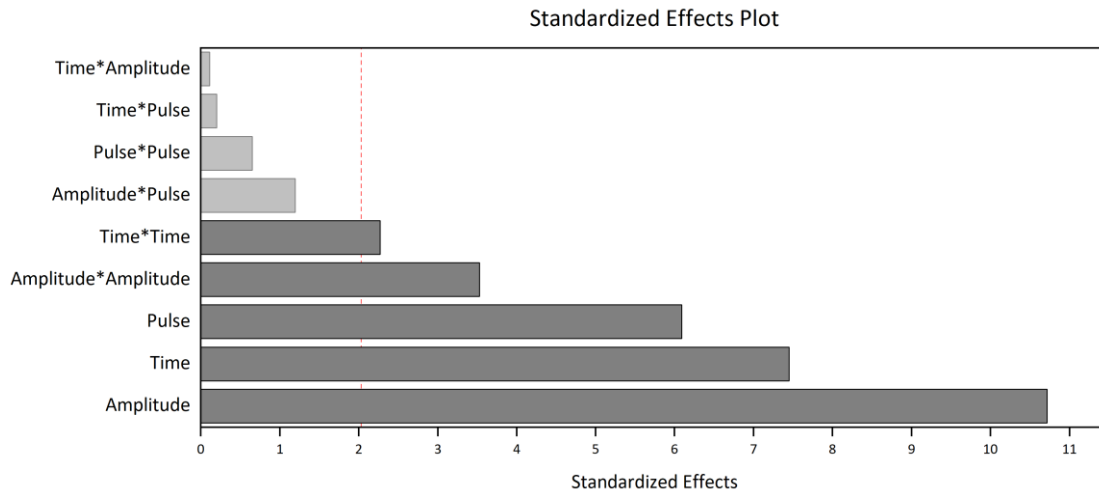


Figure 9: Standardized effects plot of the Box–Behnken response-surface model for Me:Th extraction using the ultrasonic probe.

Bars represent the standardized effects of all linear, interaction, and quadratic terms on the fatty acid yield. The vertical dashed line indicates the significance threshold ($\alpha = 0.05$). Dark bars denote significant effects, highlighting the dominant influence of extraction time and the interaction between time and temperature, while lighter bars represent non-significant factors.

The main effects plots (Figure 10) shows that extraction time increased the predicted yield steadily, with longer sonication enhancing the mechanical disruption produced by the probe. Amplitude exhibited the steepest rise among the three factors: increasing the amplitude from 40 % to around 80–90 % led to substantial improvements in extraction efficiency, after which the curve began to level off, indicating an approach toward a practical upper limit. The pulse setting also showed a positive trend, with higher pulse percentages resulting in higher predicted yields, although the increase was more gradual than for amplitude. Together, these profiles confirm that all three operational parameters directly contribute to enhanced lipid release, with amplitude exerting the strongest individual influence, followed by time and pulse.

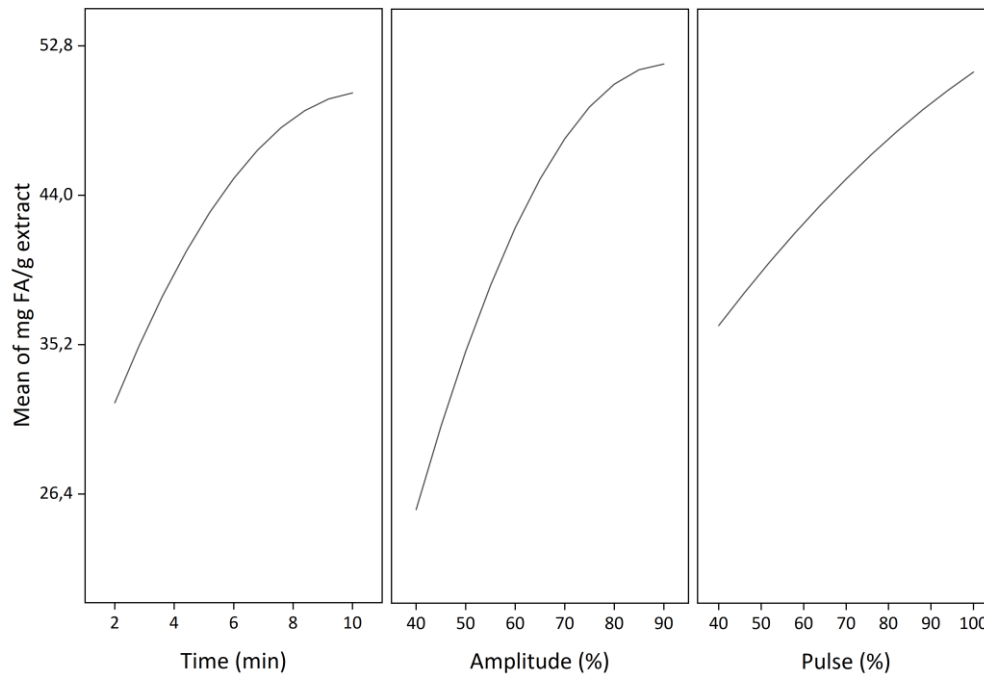


Figure 10: Main effects plots for extraction time, temperature, and pulse in the Box–Behnken model of Me:Th extraction using the ultrasonic probe.

The interaction plots (Figure 11) illustrate how pairs of factors jointly influence the fatty acid yield in the probe-assisted extraction. In all panels, the predicted yields increase consistently with higher levels of the primary factor on the x-axis, and the three lines representing the levels of the second factor run largely parallel. This indicates that the combined effects of the factors are predominantly additive: the second factor shifts the overall yield upward, but it does not substantially change the strength or direction of the first factor's effect. Across all panels, the interaction with the lowest level of the second factor consistently produced the weakest extraction performance, followed by a marked increase when moving to the intermediate level. In contrast, the additional gain from the intermediate to the highest level was smaller, suggesting a reduced additional benefit at higher settings.

This pattern was consistent across almost all interactions, however, combinations in which pulse served as the secondary factor showed a different behavior. In these panels, the three lines were more evenly spaced and remained largely parallel, demonstrating that pulse mainly acts as a uniform level shift rather than altering how strongly the primary factor influences the extraction outcome. Overall, the interaction plots demonstrate that probe-assisted extraction is mainly driven by strong main effects of amplitude, time, and pulse, while interactions between these parameters are moderate and primarily reflected as vertical level shifts rather than changes in trend or direction.

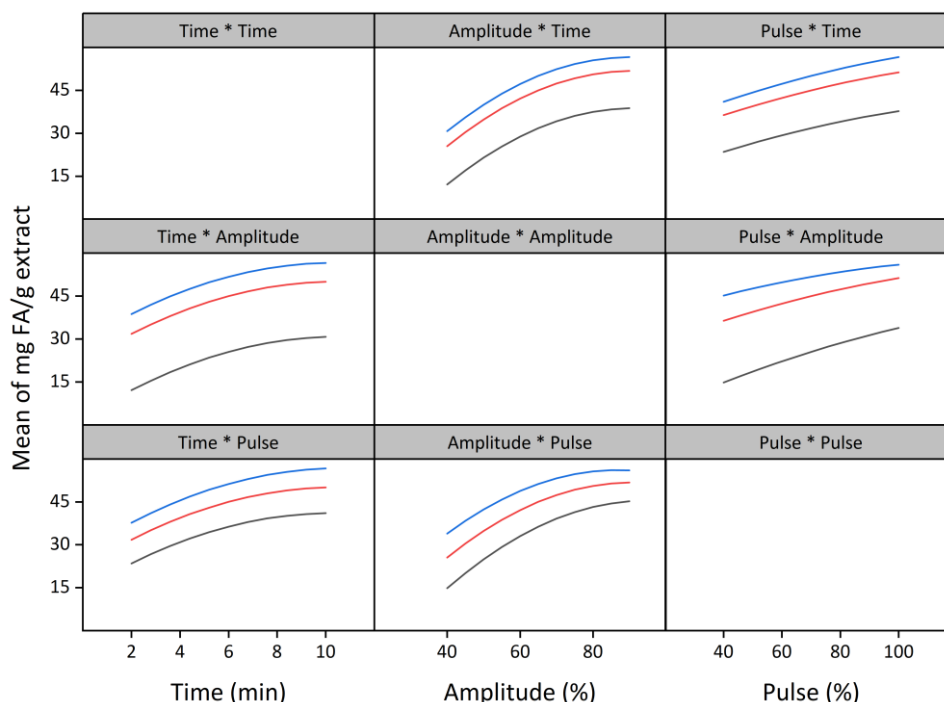


Figure 11: Two-factor interaction plots for the Box–Behnken response-surface model of Me:Th extraction using the ultrasonic probe.

In each panel, the x-axis represents the primary factor while the three colored lines depict the predicted response at the low (black), center (red), and high (blue) level of the second factor involved in the interaction.

Before analyzing the contour and surface plots, it is important to note that they are generated by holding the third factor at the optimal level predicted by the RSM model. The optimization identified 10 min extraction time, 100 % amplitude, and a pulse of 100 % as the best-performing conditions. This allows the plots to clearly show how the two changing factors work together near the predicted optimum and how they influence the yield.

The contour plots (Figure 12) reflect the consistent behavior of the probe-assisted extraction system: all three factors positively influence the fatty acid yield across their range. In each plot, the color gradient shows a smooth transition from low to high yields, indicating that higher settings of both parameters lead to improved extraction. Although the overall surface shape is similar across all factor combinations, the absolute yield range differs between plots. When time or pulse was held constant, the predicted yields spanned a broad interval from approximately 20 to 61 mg FA/g extract, whereas fixing amplitude resulted in a narrower and consistently higher range (about 32 to 61 mg FA/g extract). This difference in the lower yield values shows how strongly amplitude contributes to the extraction compared with time and pulse. The contour lines further reveal mild curvature along both amplitude–pulse

and amplitude–time interactions, indicating that increases in pulse or time become relevant only when amplitude exceeds approximately 70–75 %. Overall, the contour plots effectively illustrate the relative contributions of the individual factors and highlight the region of optimal extraction conditions.

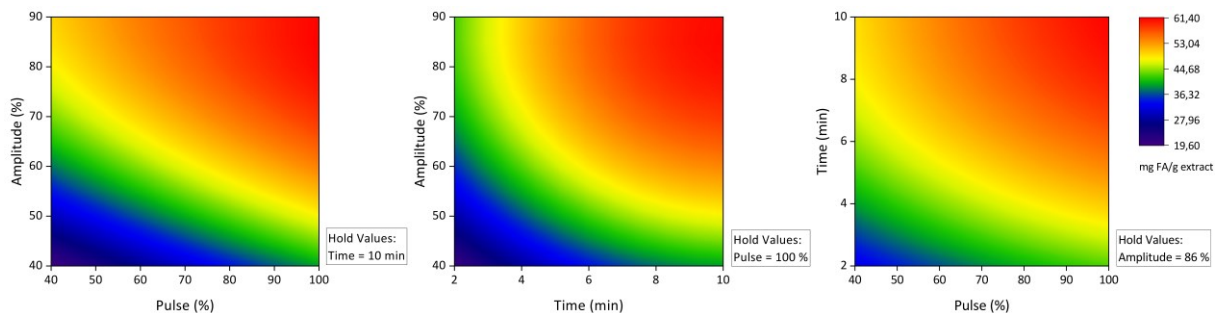


Figure 12: Contour plots of the Box–Behnken response-surface model for Me:Th extraction using the ultrasonic bath.

(a) Interaction between amplitude and pulse (time held constant at 10 min); (b) Interaction between amplitude and time (pulse held at 100 %); (c) Interaction between time and pulse (amplitude held at 86 %).

The 3D surface plots (Figure 13) add complementary information by visualizing the curvature and steepness of the response surfaces in three dimensions. They clearly show that the response increases sharply with amplitude, confirming a strong linear–quadratic effect, while the surfaces progressively flatten toward the upper amplitude range, indicating saturation of cavitation efficiency. Time contributes to a gradual elevation of the surface, particularly under high amplitude, whereas pulse generates only gentle modulations of the slope. The 3D plots also make it very clear where the global optimum is located: at high amplitude, high pulse, and long extraction time.

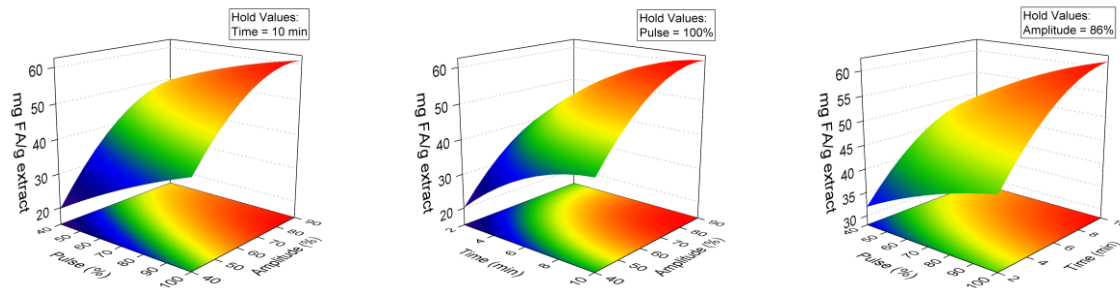


Figure 13: Three-dimensional response surface plots for the Box–Behnken model of Me:Th extraction using the ultrasonic probe.

(a) Interaction between amplitude and pulse (time held constant at 10 min); (b) Interaction between amplitude and time (pulse held at 100 %); (c) Interaction between time and pulse (amplitude held at 86 %).

Based on the full dataset of 45 extractions, the response-surface model was used to identify the parameter combination that maximizes the highest fatty acid yield in the ultrasonic probe system. The optimized extraction conditions identified by the model were: 10 min extraction time, 86.3 % amplitude, and 100 % pulse, resulting in a predicted yield of 61.3 mg FA/g extract with a composite desirability of 0.856. For experimental execution, amplitude was rounded to 86 %.

Validation experiments performed under these optimized conditions resulted in a mean yield of 54.32 ± 1.73 mg FA/g extract ($n = 3$). This value falls within the 95 % confidence interval of the model prediction (53.71–68.90 mg FA/g extract), indicating good agreement between predicted and observed performance, although the experimental yield remained at the lower boundary of the expected range.

The probe-assisted extraction clearly outperformed the ultrasonic bath in terms of extraction efficiency, parameter sensitivity, and model explanatory power. While the bath system produced a relatively broad and stable operating window with moderate yields, the probe system generated substantially higher lipid release within significantly shorter extraction times. This difference reflects fundamental physical distinctions between both ultrasound sources: the probe delivers localized, high-intensity cavitation directly into the medium, whereas bath transducers create a weaker and spatially diffuse acoustic field. The statistical models reinforce these mechanistic differences. The probe model showed a markedly higher R^2 (0.8566 vs. 0.5003 in the bath), indicating that most of the variability could be captured by the tested parameters. In contrast, the bath model displayed lower predictive power and a broader distribution of yields across the design space, suggesting that extraction performance in the bath is governed by slower, more diffusion-controlled mechanisms. The stronger

quadratic effects in the probe model also indicate that very high amplitudes eventually become less efficient, likely due to bubble coalescence and localized heating-effects that are much weaker in the bath system.

However, the greater efficiency of the probe is accompanied by higher variability. Small changes in probe position, biomass distribution around the tip, or local temperature fluctuations can noticeably affect extraction performance. The bath system, although less powerful, produces a more uniform acoustic field and therefore produces more consistent results across repeated runs. In practice, this means that the bath offers lower yields but greater reproducibility, while the probe achieves higher yields in significantly less time but requires tighter control of operating conditions.

Overall, the comparison shows that probe-assisted UAE with Me:Th is the more powerful extraction method, capable of achieving high lipid yields in only 10 minutes. However, it requires stricter operational control to maintain reproducibility. The bath system remains advantageous when robustness and ease of handling are prioritized, while the probe system is better suited for applications where maximized extraction efficiency is critical and process control can be tightly maintained.

5.1.3.1 Temperature–time profiles during probe-assisted ultrasound extraction

To investigate the thermal effects associated with probe-assisted UAE, temperature–time curves were recorded for all parameter combinations of the Box–Behnken design. Although all extractions were performed on ice to counteract heat buildup, the high acoustic energy released by the probe led to a measurable increase in the temperature of the Me:Th phase. For clarity, the temperature profiles are grouped into three sets of graphs, each representing experiments in which one parameter (time, amplitude, or pulse) was held constant at its coded levels (-1, 0, +1). These coded levels correspond to low, middle, and high settings, specifically 2/6/10 min for extraction time, 40/65/90 % for amplitude, and 40/70/100 % for pulse. In the temperature profiles, “T”, “A”, and “P” denote extraction time (min), amplitude (%), and pulse (%), respectively (e.g., T2/A90/P70 corresponds to 2 min, 90 % amplitude, 70 % pulse). This structure allows direct comparison of how each factor influences heating dynamics and reveals how ultrasonic intensity and duty cycle shape the thermal behavior of the extraction system.

In the 2-min temperature profile (Figure 14, left), the high-amplitude/middle-pulse (A90–P70) and middle-amplitude/high-pulse (A65–P100) curves show the most pronounced temperature rise, with both increasing rapidly during the first 40–50 seconds, although the A65–P100 curve levels off earlier. The middle-amplitude/low-pulse (A65–P40) and low-amplitude/middle-pulse (A40–P70) curves

remain close to the initial temperature throughout the measurement period, showing only minimal fluctuations.

In the 6-min temperature profile (Figure 14, middle), the high-amplitude/high-pulse condition (A90–P100) reaches the highest temperatures and shows the steepest overall increase. The high-amplitude/low-pulse (A90–P40) and middle-amplitude/middle-pulse (A65–P70) combinations show moderate heating, while low-amplitude/high-pulse (A40–P100) rises more slowly; the low-amplitude/low-pulse curve (A40–P40) remains near the baseline.

In the 10-min temperature profile (Figure 14, right), the middle-amplitude/high-pulse (A65–P100) and high-amplitude/middle-pulse (A90–P70) curves reach similar upper temperature plateaus, clearly exceeding the other conditions. The low-amplitude/middle-pulse (A40–P70) and middle-amplitude/low-pulse (A65–P40) combinations show slower and more moderate temperature increases that level off at lower final temperatures.

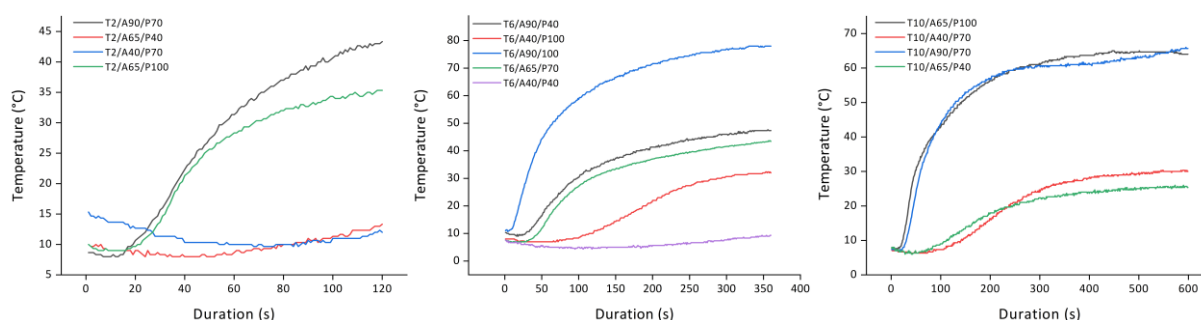


Figure 14: Temperature development during ultrasonic extraction at 2 min (left), 6 min (middle), and 10 min (right)

The temperature measurements showed that heating during probe-assisted UAE depends strongly on the specific combination of amplitude and pulse. Noticeable temperature increases occurred not only under high–high conditions, but also under middle–high and high–middle settings, while middle–middle and middle–low combinations produced moderate heating. The only condition that remained close to the baseline was the low–low setting, indicating that both acoustic intensity and duty cycle must reach at least middle levels to generate substantial thermal energy.

Across the three time settings, the same pattern became stronger with increasing sonication duration. At short extraction times, temperature rises were limited to the most intense combinations, whereas at 6 and 10 minutes, the differences between the curves became wider and more pronounced. All heating curves eventually reached a plateau, showing that the system stabilizes once the balance

between ultrasound energy input and heat dissipation is reached. This demonstrates that the Me:Th NaDES heats up quickly when cavitation intensity is sufficient but does not continue to rise indefinitely.

These temperature effects are relevant for extraction efficiency. Higher temperatures reduce the viscosity of the hydrophobic NaDES and improve mass transfer, which can support faster release and solubilization of lipids. At the same time, excessive temperatures may pose a risk for thermally sensitive compounds, especially long-chain polyunsaturated fatty acids. This highlights the importance of identifying operating conditions that enhance extraction without overheating the system.

Although all experiments were carried out on ice, the strong heating during high-intensity sonication confirms how energetically demanding the probe system is. The initial drop seen in some curves can be attributed to the fact that the extraction was started immediately, before the NaDES temperature had fully equilibrated with the ice bath. This also explains some of the variability observed between runs with the same settings. Small differences in probe positioning, contact with the vessel wall, local cavitation patterns, or temperature equilibration can noticeably shift the thermal profiles.

Overall, the temperature curves show that the heating behavior of the probe system is defined by the combined effect of amplitude and pulse, strengthened by extraction time, and shaped by the balance between rapid initial heating and plateau formation. These findings are important for understanding the extraction mechanism and for setting practical limits to avoid unnecessary overheating while still benefiting from the positive effects of moderate temperature increases.

5.1.3.2 Temperature evolution under optimized conditions: Comparison of NaDES and hexane

Additionally, a direct comparison of NaDES and n-hexane under identical optimized probe-assisted extraction conditions (10 min, 86 % amplitude, 100 % pulse) shows that the two solvents exhibit markedly different temperature profiles (Figure X). The Me:Th NaDES exhibits a rapid temperature increase during the first 60–90 s, followed by a gradual rise toward a plateau of approximately 65–70 °C, where the curve remains relatively stable for the remainder of the extraction. In contrast, hexane heats up more slowly and reaches only moderate temperatures, rising steadily to about 25–28 °C without forming a pronounced plateau. The two curves separate early in the process and maintain a large temperature difference throughout the entire sonication period.

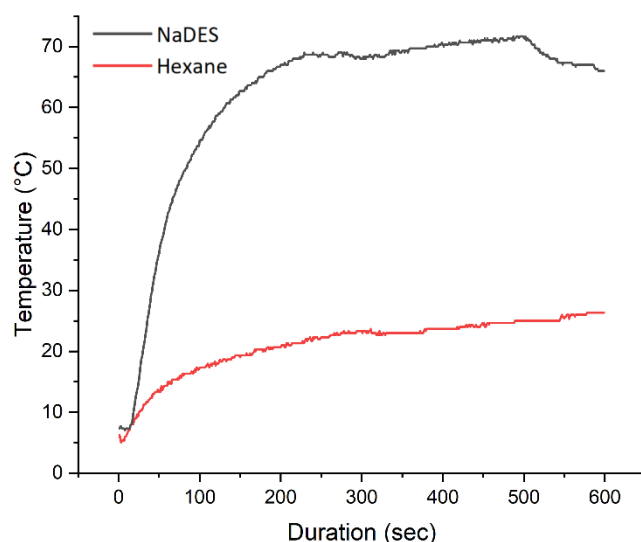


Figure 15: Extraction with NaDES and Hexane under best conditions

The strong difference in temperature development between NaDES and n-hexane under identical probe-assisted extraction conditions can be largely explained by the contrasting viscosities of the two solvents. The Me:Th NaDES, which is considerably more viscous than hexane, absorbs ultrasonic energy much more efficiently: the acoustic input is converted into frictional heat, leading to the rapid temperature rise observed during the first 60–90 seconds. In contrast, the very low viscosity of hexane results in minimal dissipation of ultrasound energy, which is reflected in its slow and nearly linear heating profile.

This viscosity effect also explains the formation of a clear thermal plateau for NaDES but not for hexane. As the viscous NaDES warms up, it reaches a temperature where heat generation and heat loss balance each other, stabilizing at approximately 65–70 °C. Hexane, due to its low viscosity and higher thermal conductivity, dissipates heat more effectively and therefore does not reach a comparable steady state. These thermal differences directly influence extraction performance: for NaDES, the decrease in viscosity at elevated temperatures enhances mass transfer, lipid diffusion and solubilization, supporting the high yields achieved under probe sonication. Hexane, whose viscosity changes little with temperature, benefits far less from thermal acceleration. On the other hand, hexane's lower temperature profile reduces the risk of thermal degradation of sensitive lipids, whereas NaDES operates closer to conditions where heat-induced oxidation of polyunsaturated fatty acids becomes plausible. Therefore, although the stronger heating of NaDES contributes to its higher extraction efficiency, it

also means that temperature must be controlled more carefully than with hexane to prevent any loss in lipid quality.

5.1.4 Reference extraction with *n*-hexane (Soxhlet and UAE)

To establish a benchmark for lipid recovery from *Schizochytrium* biomass, Soxhlet was used as the traditional procedure for obtaining a reference lipidic yield. After complete evaporation of the solvent, the resulting extract yielded 27.76 ± 0.15 %, and GC-MS analysis of the dried extract quantified 545.4 ± 37.7 mg FA/g extract.

To compare this conventional reference with ultrasound-assisted extraction, hexane was subsequently used in the ultrasonic bath under the same conditions applied for the NaDES experiments (30 min, 30 °C). Here again, the hexane was fully evaporated after extraction, and the lipid yield was determined gravimetrically, resulting in 34.63 ± 1.04 %.

For a direct comparison with NaDES-based UAE under optimized conditions, hexane was finally tested using the best RSM-derived settings for each ultrasound system separately. In the bath, hexane extractions were carried out under the bath-optimized NaDES conditions, and in the probe system under the probe-optimized NaDES conditions. In these experiments, the solvent was not evaporated; instead, GC-MS quantification was performed on the extract-solvent mixtures, analogous to the NaDES protocol, where phase separation is not possible. Under the bath-optimized conditions, hexane-based UAE yielded 49.22 ± 2.58 mg FA/g extract, while under the probe-optimized conditions the value increased to 52.33 ± 0.37 mg FA/g extract.

The Soxhlet yield obtained in this work is consistent with previously reported values for *Schizochytrium* biomass, where a Soxhlet extraction efficiency of 24.04 ± 0.25 % has been documented [18]. Lipid yields obtained with the Folch method report lipid yields between 10 and 45 % [70]. It should be noted that lipid content in *Schizochytrium* is highly dependent on cultivation conditions, nutrient availability and seasonal variability, meaning that absolute extraction yields may differ between studies even when using identical methods [1].

When hexane was used under the NaDES-optimized UAE settings, the resulting fatty acid concentrations amounted to 49.22 ± 2.58 mg FA/g extract in the bath and 52.33 ± 0.37 mg FA/g extract in the probe. These values can be directly compared to the corresponding NaDES experiments, which yielded 55.85 ± 0.44 mg FA/g extract in the bath and 54.32 ± 1.73 mg FA/g extract in the probe. The consistently

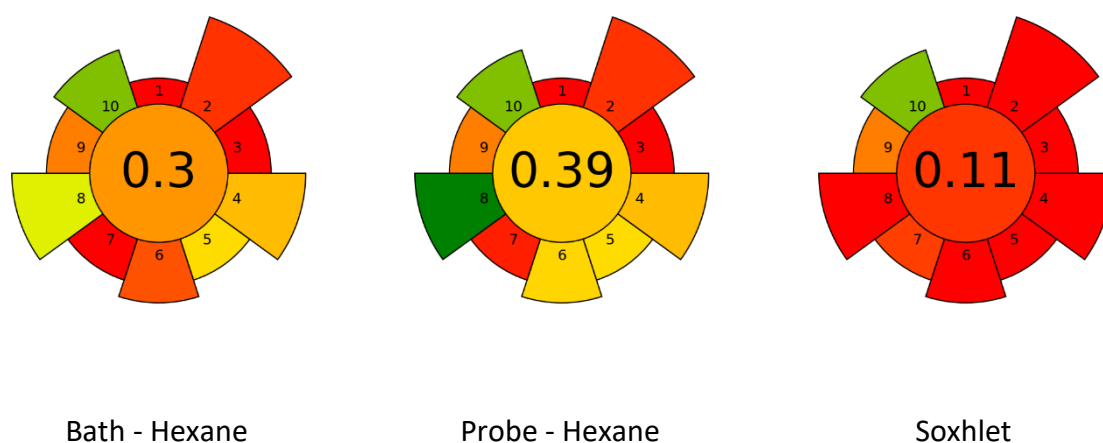
lower FA concentrations obtained with hexane indicate that, although ultrasound enhances lipid release in both solvents, hexane benefits far less from the intensified ultrasound conditions. This outcome aligns with the solvent-specific ultrasound behavior observed in the temperature measurements: hexane remains comparatively cool and shows limited cavitation intensity due to its low viscosity, whereas NaDES undergoes pronounced heating and viscosity reduction, which improves energy coupling, cell disruption, and mass transfer. Consequently, NaDES provides a more responsive extraction environment under high-intensity UAE, leading to higher fatty acid yields than n-hexane under identical operating conditions.

5.1.5 Selection of the optimal extraction strategy

To select the optimal extraction method, two parameters were evaluated: (i) the greenness of the method and (ii) the results expressed as mg FA/g extract. This two-step approach ensures that the final choice reflects both environmental and analytical performance.

5.1.5.1 AGREEprep sustainability assessment

To compare the environmental sustainability of the extraction protocols developed in this study, five methods (Soxhlet, hexane-bath UAE, hexane-probe UAE, NaDES-bath UAE, NaDES-probe UAE) were evaluated using the AGREEprep tool. Figure 16 summarizes the resulting greenness scores, based on the ten principles of green sample preparation.



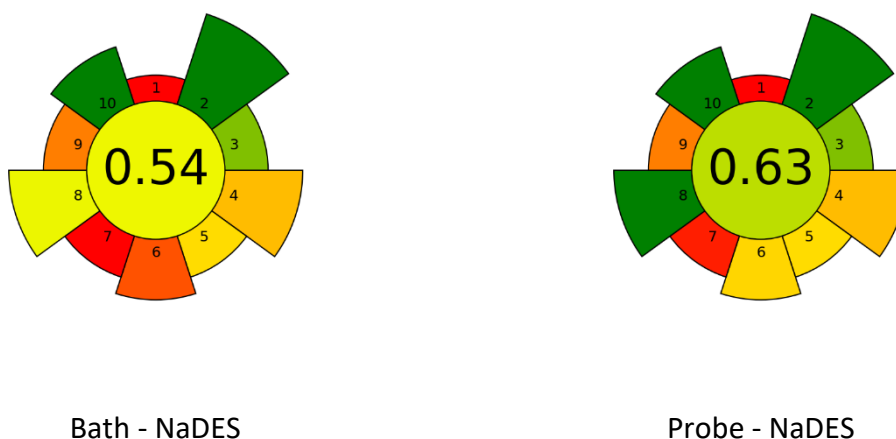


Figure 16: Agreeprep diagrams for the five extraction methods evaluated in this study.

Soxhlet (hexane, 6 h, continuous reflux), bath–hexane UAE (30 min, 30 °C), probe–hexane UAE (10 min, 86 % amplitude, 100 % pulse), bath–NaDES UAE (Me:Th = 1:1, 30 min, 30 °C), and probe–NaDES UAE (Me:Th = 1:1, 10 min, 86 % amplitude, 100 % pulse).

Among the conventional approaches, Soxhlet extraction displayed the lowest score (0.11), which reflects its intensive solvent consumption, long extraction time with continuous heating, and low degree of automation. Hexane-based ultrasound-assisted extraction performed moderately better, reaching scores of 0.30 (bath) and 0.39 (probe). These improvements result from shorter extraction times and reduced energy demand relative to Soxhlet, although the reliance on a volatile solvent remains a major limitation.

In contrast, the NaDES-based protocols demonstrated significantly better performance. The bath method using NaDES achieved a score of 0.54, and the probe-based method reached the highest score of 0.63. These improvements are mainly attributable to the replacement of volatile organic solvents with safer, renewable NaDES components. The superior performance of the ultrasound probe is mainly attributed to several operational advantages over the bath. Firstly, the probe system enabled the processing of up to six samples per hour, whereas the bath setup was limited to only two, thus increasing sample throughput. Secondly, the probe protocol required fewer discrete handling steps (five versus more than six for the bath), which reduces manual effort and the potential for error. Lastly, the energy consumption of the probe system was markedly lower, requiring approximately 5 Wh per run, in contrast to about 60 Wh for the bath setup, which operated with continuous heating over an extended time period. Importantly, these differences remained consistent regardless of whether hexane or NaDES was used as the extraction solvent, highlighting that not only the solvent type but also the instrumental setup critically impacts the greenness of the method.

5.1.5.2 Comparison of experimental extraction performance

Since both ultrasound strategies (bath and probe) showed the best performance in terms of the greenness of the method, the yields obtained for both were compared to decide on the optimal method to use for future work. Under their respective optimized conditions, the NaDES-based ultrasound bath yielded 55.85 ± 0.44 mg FA/g extract, while the ultrasonic probe produced 54.32 ± 1.73 mg FA/g extract. Although these values are very similar and an ANOVA confirmed that the difference between the two methods was not statistically significant, additional operational factors supported the selection of the probe setup as the preferred method. Compared with the bath, the probe offered higher sample throughput, fewer handling steps, and markedly lower energy consumption, while maintaining comparable extraction performance and reproducibility.

Based on the combination of greenness, operational efficiency, and experimental extraction performance, the ultrasonic probe was therefore selected as the optimal method for subsequent NaDES screening and further investigations.

5.2 Lipid extraction using fatty acid-based NaDES: Recovery strategies

The hydrophobic Me:Th NaDES used in the previous experiments showed good extraction performance but did not allow any post-extraction separation of the solvent from the lipid fraction. This limitation motivated the exploration of alternative NaDES systems that have been reported in the literature to undergo reversible changes in hydrophobicity, enabling phase separation after extraction [71].

Fatty-acid-based NaDES represent a promising class of such systems. Previous studies have shown that mixtures of medium-chain fatty acids, such as octanoic and decanoic acid, form stable hydrophobic eutectics with low viscosity, low density and very limited water miscibility, making them among the most hydrophobic NaDES described so far [13]. Their components are structurally related to the target lipids, and studies have demonstrated that their polarity can be modulated through protonation, deprotonation or interaction with amine-based phase-switching agents, enabling the selective recovery of non-polar compounds [44].

In this chapter, fatty-acid-based NaDES were characterized with respect to their physicochemical properties and evaluated for their ability to extract lipids from *Schizochytrium* while permitting post-

extraction phase separation. Particular attention was given to recovery strategies based on amine-induced hydrophilization, which have been highlighted as an effective route for separating fatty-acid-rich NaDES from lipid products. The following sections describe the preparation, characterization and extraction behavior of these NaDES systems and assess their potential as recoverable green solvents.

5.2.1 Physicochemical characterization of fatty-acid-based NaDES

Various NaDES combinations were tested at different molar ratios to assess their physical stability. All mixtures were prepared at 40 °C to ensure complete melting of the components and to facilitate hydrogen-bond formation during NaDES assembly. After 30 min of mixing, the systems C8:C12 (3:1), C8:Me (1:1, 3:1), C8:Th (1:1, 3:1), and C8:Ca (3:1) formed homogeneous and stable liquids at room temperature and were selected for further work.

Mixtures containing C12 with Me or Th at a 1:1 ratio were physically unstable, displaying phase separation or partial solidification. Adjustment of the molar ratio to 2:1 (Me/Th:C12) led to stable liquids; however, these systems were excluded from further analysis because preliminary phase-switching experiments showed that Jeffamine could not induce reversible hydrophilization to a satisfactory extent.

The physicochemical characterization focused on those NaDES combinations that were both stable and used in the extraction experiments. Their densities were determined gravimetrically at room temperature. An overview of the selected NaDES systems, their molar ratios and density values is presented in Table 3.

Table 3: Fatty-acid-based NaDES systems used in this study, their molar ratios and measured densities at room temperature (mean $\pm$ SD, $n = 3$).

NaDES	Molar ratio	Density (g/mL)
C8:C12	3:1	0.908 $\pm$ 0.001
C8:Me	3:1	0.909 $\pm$ 0.001
C8:Th	3:1	0.928 $\pm$ 0.001
C8:Ca	3:1	0.931 $\pm$ 0.001

The measured densities ranged from 0.908 g/mL (C8:C12, 3:1) to 0.931 g/mL (C8:Ca, 3:1). As expected, systems containing medium-chain fatty acids such as C8 and C12 showed lower density values

compared to those composed of menthol and thymol. This is consistent with previous reports indicating that fatty-acid-based NaDES generally exhibit lower densities than terpene-based systems due to their lower molecular weight and less rigid molecular packing [13].

5.2.2 Extraction performance and Jeffamine-assisted phase-switching recovery

Based on the previously optimized probe conditions (10 min, 86 % amplitude, 100 % pulse), four fatty-acid-based NaDES systems were applied for lipid extraction from *Schizochytrium* biomass and subsequent Jeffamine-assisted phase switching. These NaDES were specifically selected to enable recovery of fatty acids, as their hydrophobic and fatty-acid-based composition allows reversible phase switching, in contrast to menthol:thymol systems where no effective phase separation could be achieved. Apparent extraction yields (% w/w relative to biomass) and fatty acid contents (mg FA/g extract) are summarized in Table 5 together with Soxhlet extraction as a reference method. To improve data consistency and minimize the effect of injection variability, quantification of mg FA/g extract was performed using the averaged peak area of the internal standard. This approach significantly reduced the standard deviation, while maintaining the relative trends between extractions.

Soxhlet extraction, which is expected to recover mainly neutral lipids, resulted in a yield of 27.76 ± 0.15 % and a fatty acid concentration of 552.82 ± 38.14 mg FA/g extract. Compared with this reference, all NaDES systems gave higher apparent yields, ranging from 35.96 ± 1.49 % (C8:C12) to 117.19 ± 3.95 % (C8:Me). Since it is not physically possible to recover more than the total mass of extractable lipids from the biomass, these values above the Soxhlet yield indicate that part of the recovered material is not lipid but residual NaDES that remained in the extract after the Jeffamine-induced phase inversion. When the hydrophilization is not fully effective, a fraction of the NaDES does not transition into the aqueous Jeffamine phase and instead remains in the upper organic layer. This incomplete separation is strongly system-dependent, as the ability of a NaDES to undergo polarity switching varies with the chemical nature and ratio of its individual components.

Table 4: Extraction yields and fatty acid concentrations of lipid extracts obtained with fatty-acid-based NaDES and Soxhlet extraction (mean $\pm$ SD, n = 3).

NaDES	Yield (%)	mg FA/g extract
C8:C12	35.96 ± 1.49^c	608.77 ± 22.02^a
C8:Me	117.19 ± 3.95^a	317.95 ± 12.64^c
C8:Th	50.33 ± 1.39^b	522.92 ± 40.04^b

C8:Ca	51.35 ± 0.52^b	497.55 ± 8.89^b
Soxhlet	27.76 ± 0.15^d	552.82 ± 38.14^{ab}

Among the NaDES systems, C8:Me exhibited by far the highest apparent yield (117.19 %), followed by C8:Th and C8:Ca with intermediate values of 50.33 % and 51.35 %, respectively, whereas C8:C12 gave the lowest yield (35.96 %). Since extraction yields exceeding 100 % are physically not plausible, this value clearly indicates substantial co-extraction and incomplete phase separation, leading to residual NaDES being present in the recovered extract. This pattern suggests that C8:Me is most prone to incomplete separation during the phase-switching step, while C8:C12 shows the cleanest separation between the NaDES-rich and lipid-rich phases. Therefore, the apparent yield of the C8:Me system cannot be interpreted as a true extraction efficiency, but rather reflects limitations in the recovery process. To further examine whether the unusually high yield of the C8:Me system could be reduced through additional purification, a second Jeffamine-based phase-separation step was performed. However, this resulted in only a minor decrease of 10.28 % in extract mass, confirming that significant amounts of NaDES remain with the extract even after repeated separation attempts.

To differentiate total recovery from extract purity, the fatty acid contents were additionally expressed as mg FA/g extract. When considering this metric, the picture changes markedly. C8:Me, despite its very high apparent yield, showed the lowest fatty acid concentration (317.95 ± 12.64 mg FA/g extract), confirming that a substantial fraction of the recovered material consists of non-lipid components. C8:Th and C8:Ca reached intermediate values of 522.92 ± 40.04 and 497.55 ± 8.89 mg FA/g extract, which are close to the Soxhlet reference (552.82 ± 38.14 mg FA/g extract), indicating that these extracts still contain some NaDES components, but to a lesser extent.

In contrast, C8:C12 combined the lowest apparent yield with the highest fatty acid concentration (608.77 ± 22.02 mg FA/g extract), exceeding the Soxhlet value. This indicates that, although less mass was recovered overall, the extract obtained with C8:C12 was the most enriched in lipids and contained the smallest proportion of co-extracted NaDES. Taken together, the comparison of yield (%) and mg FA/g extract demonstrates that the yield values mainly reflect the efficiency of the phase separation rather than the true lipid yield, and that C8:C12 provides the most selective and “clean” extraction among the tested NaDES systems.

5.2.3 Compositional analysis of the recovered extracts (GC–MS)

While total yield (%) and fatty acid contents (mg FA/g extract) provide an overview of recovery and extract purity, they do not reveal the detailed composition of the recovered material. To assess the selectivity of each NaDES system and quantify the presence of residual solvent components after phase switching, the extracts were analyzed by GC–MS. The relative abundances of fatty acids and remaining NaDES constituents are shown in Figure X. Figure 17.

Before comparing the extracts obtained with the different NaDES systems, the lipid profile of *Schizochytrium* oil obtained via Soxhlet extraction was used as a reference. Literature data consistently report palmitic acid (C16:0) at approximately 18–19 %, EPA at around 16 %, and DHA as the dominant fatty acid with ~34 % of total lipids [72]. These proportions align well with the present Soxhlet results, which also show DHA as the major constituent, followed by EPA and C16:0. Minor fatty acids, some occurring at levels up to approximately 4 %, were not included in the graphical representation, as the comparison focuses on the principal constituents relevant for evaluating NaDES selectivity. As a consequence, the relative percentages of DHA, EPA, and C16:0 in the stacked-bar figure may appear slightly elevated due to the omission of these lower-abundance components. Nevertheless, the Soxhlet profile reliably reflects the characteristic fatty acid distribution of *Schizochytrium sp.* serves as a benchmark for assessing the fatty acid composition of NaDES-derived extracts.

Across all samples, no detectable residues of octanoic acid (C8) were observed in the GC-MS chromatograms. This indicates that C8 was consistently and completely separated during the Jeffamine-induced polarity switching, irrespective of its role as NaDES component.

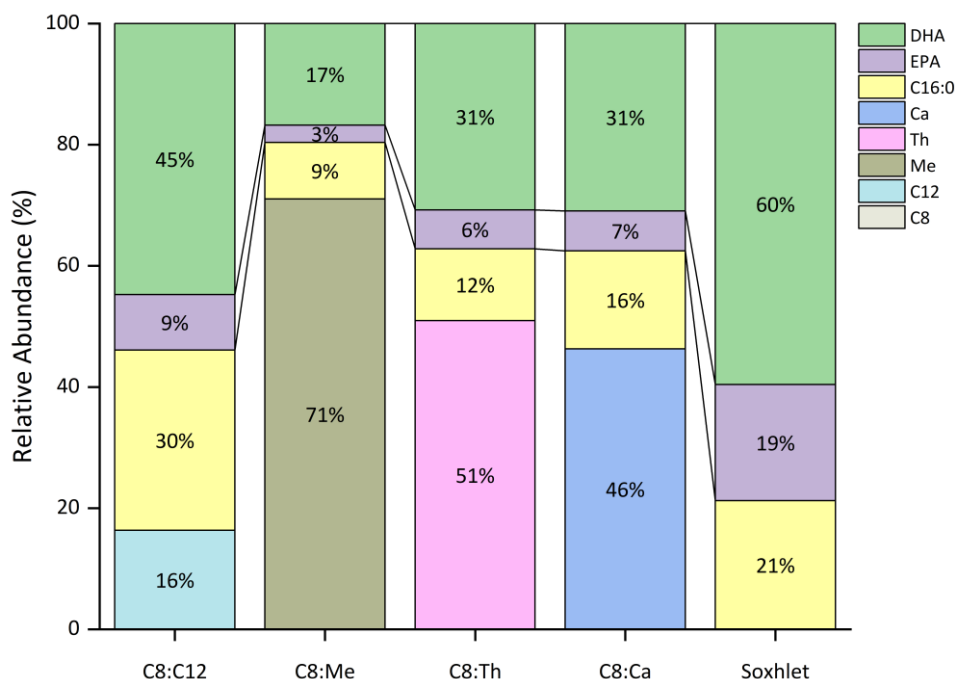


Figure 17: Relative composition (%) of recovered extracts obtained with fatty-acid-based natural deep eutectic solvents (NaDES; C8:C12, C8:Me, C8:Th, C8:Ca) compared with Soxhlet extraction.

Values represent the GC-MS-derived relative abundance of the major fatty acids (docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and palmitic acid (C16:0)) and residual NaDES components (octanoic acid (C8), dodecanoic acid (C12), menthol (Me), thymol (Th), and carvacrol (Ca)).

The compositional profiles highlight pronounced differences in separation efficiency between the systems. The C8:C12-based NaDES produced the cleanest extract, containing predominantly DHA (45 %), C16:0 (30 %), and EPA (9 %) accounting for the majority of the recovered phase, while only 16 % of C12 remained. This agrees with its high mg FA/g extract value and confirms that C8:C12 underwent the most efficient polarity switch during Jeffamine treatment.

In contrast, the C8:Me-based NaDES showed a markedly different profile. The extract consisted mainly of menthol (71 %), whereas the fatty acid fraction was limited to DHA (17 %), C16:0 (9 %) and EPA (3 %). This composition directly explains the extreme apparent yield (117 %) and the very low mg FA/g extract, demonstrating that the high mass recovery was caused by large amounts of unremoved menthol rather than improved lipid extraction.

The C8:Th-based NaDES exhibited partial selectivity: thymol accounted for 51 % of the extract, accompanied by moderate proportions of DHA (31 %), C16:0 (12 %), and EPA (6 %). Similarly, the C8:Ca-based NaDES retained considerable amounts of carvacrol (46 %) in the extract, with DHA (31 %), C16:0 (16 %)

%), and EPA (7 %) forming the remaining lipid fraction. These results indicate incomplete phase inversion and are consistent with the intermediate values obtained for yield and mg FA/g extract.

Taken together, the compositional analysis clearly identifies the C8:C12-based NaDES as the most selective system, providing the highest degree of solvent–lipid separation and yielding an extract closely resembling native *Schizochytrium* oil. The C8:Me-based NaDES performed poorest due to extensive solvent retention, while the C8:Th- and C8:Ca-based NaDES systems exhibited intermediate behavior, suggesting that additional purification steps would be required if solvent-free lipid extracts are desired.

5.2.4 Comparative evaluation of fatty-acid-based NaDES extraction and recovery

The comparative evaluation of fatty-acid-based NaDES systems demonstrated that apparent extraction yield is not a reliable indicator of extraction efficiency, as it is strongly influenced by solvent retention during phase switching. Instead, the combination of fatty acid concentration and compositional analysis revealed that C8:C12 provides the most effective recovery, with minimal NaDES carryover and the highest extract purity. In contrast, terpene-containing systems showed incomplete phase separation due to the absence of fully switchable components, resulting in residual solvent in the lipid-rich phase. Overall, the findings clearly demonstrate that switchable recovery works highly effectively for NaDES that contain protonatable fatty acids, while terpene-based systems undergo only partial removal, a predictable and chemically consistent outcome. This highlights an important design criterion: NaDES intended for reversible extraction should include at least one component capable of undergoing a polarity shift. The study therefore provides not only practical extraction data, but also a deeper understanding of how molecular structure governs recovery efficiency, offering valuable guidance for future NaDES development.

5.3 Cytotoxicity of NaDES: extracts, pure NaDES and individual components

While the extraction results show how efficiently the NaDES systems recover and separate lipids, they do not reveal whether the remaining solvent traces are biologically safe. Even small amounts of residual components can affect cellular responses. Evaluating cytotoxicity is therefore a crucial next step, as it complements the physicochemical and compositional data and allows a more complete assessment of the overall suitability of the NaDES systems. In the following chapter, cytotoxicity is examined on three levels: first in the recovered extracts, then in the pure NaDES mixtures, and finally in the

individual NaDES components. This tiered approach enables a holistic interpretation of potential biological effects and allows the origin of any cytotoxic response to be clearly attributed.

To place the cytotoxicity results into context, it is helpful to first outline what is already known about the toxicological behavior of the individual NaDES components. These reference data provide a baseline for interpreting whether any observed effects in the following experiments reflect the extracts themselves or the intrinsic properties of the compounds. The next section briefly summarizes the relevant toxicological characteristics of each substance.

Caprylic acid shows low acute systemic toxicity, with an oral LD₅₀ of 1410 mg/kg in rats [73] and is considered safe at current intake levels as a flavoring agent [74]. *In vitro* data indicate that C8 is generally well tolerated: in HCT116 cells, only moderate effects were observed at 100 µg/mL, while pronounced cytotoxicity occurred only at substantially higher, millimolar concentrations [75]. These findings suggest that C8 exerts measurable toxicity only at elevated doses, providing a suitable reference frame for interpreting the concentrations used in this study.

Lauric acid (C12) shows very low acute systemic toxicity, with an oral LD₅₀ of 12,000 mg/kg bw in rats [73], and is considered safe at current intake levels as a flavoring agent [73]. *In vitro*, C12 exhibits a clear concentration-dependent reduction in viability in HCT116 cells: after 48 h exposure, cell viability decreased to around 60 % at 50 µg/mL [76]. These findings suggest that C12 remains well tolerated at lower concentrations and begins to exert cytotoxic effects at elevated doses.

Menthol shows low acute systemic toxicity (oral LD₅₀ ≈ 3000 mg/kg in rats) and is widely used in pharmaceutical and cosmetic formulations [77]. Regulatory evaluations classify menthol as safe for human exposure, with an acceptable daily intake (ADI) of 0-4 mg/kg body weight [78]. *In vitro*, menthol generally exhibits mild cytotoxicity, with effects occurring only at higher concentrations and in a cell-type-dependent manner. A study using peppermint essential oil containing ~22 % menthol reported no viability loss below 80 % in HCT116 cells even at an effective menthol concentration of ~112 µg/mL, indicating low cytotoxic potential within the concentration range relevant to this work [79].

Thymol shows moderate acute toxicity, with reported oral LD₅₀ values in rats around 980 mg/kg bw [80], and is considered safe at typical dietary intake levels [81]. *In vitro*, thymol exhibits clear, concentration-dependent cytotoxicity. A study using HCT116 colorectal carcinoma cells reported substantial growth inhibition after 24 h exposure, with marked viability reductions above 40–80 µg/mL [82]. These data indicate that thymol exerts stronger cytotoxic effects than menthol and the literature supports

that thymol displays measurable cytotoxicity within the concentration range examined in the present study.

Carvacrol shows moderate acute toxicity, with reported oral LD₅₀ values in rats around 810 mg/kg [83]. It is widely used as a flavoring compound and evaluated as safe at current intake levels, with no safety concern assigned by international regulatory bodies [81]. *In vitro*, carvacrol exhibits clear, concentration-dependent cytotoxicity, particularly in cancer cell lines. In HCT116 colorectal carcinoma cells, treatment with 25–200 µM carvacrol resulted in progressive reductions in cell proliferation, with viability decreasing to ~45 % at 100 µM after 24 h exposure in an xCELLigence real-time assay [84]. These findings indicate that carvacrol exerts measurable antiproliferative activity at mid-micromolar concentrations.

Jeffamine® (poly(propylene glycol) bis(2-aminopropyl ether)) is an aminated polyether used industrially as a curing agent and as a phase-switching amine in extraction processes [44]. Jeffamine exhibits moderate acute toxicity, with reported oral LD₅₀ values in rats of approximately 2885 mg/kg bw [85]. Although Jeffamine was not detected in the recovered extracts by GC–MS, its cytotoxicity was assessed for completeness because the analytical method applied in this work does not reliably detect highly polar, non-volatile polyetheramines, and trace-level residues cannot be fully excluded. Evaluating the intrinsic cytotoxicity of Jeffamine therefore provides an additional safety margin and contributes to a more comprehensive toxicological characterization of the overall NaDES extraction system.

Most available cytotoxicity data for the individual NaDES components are derived from HCT116 or other tumor cell lines, while comparable studies for HCEC-1CT are not available. For the NaDES systems, no cytotoxicity data are available that match the molar ratios, cell lines or concentration ranges used in this study. Existing studies differ in composition, exposure time, assay format and model system, which makes a direct comparison impossible. The present work therefore provides essential data for these specific NaDES combinations under defined and comparable conditions.

Viability was quantified by applying the CTB and SRB assays after 24 h exposure. Results were normalized to the respective solvent controls, and data from both cell lines (HCEC-1CT and HCT116) were displayed side-by-side for direct comparison. Statistical significance was evaluated for all treatments, and any statistically significant deviation from the control was interpreted as a biologically relevant effect. Concentrations of NaDES extracts and pure compounds are reported as treatment

concentrations ($\mu\text{g mL}^{-1}$), whereas concentrations of Triton X-100 and DMSO are expressed as volume percentages (v/v).

5.3.1 Cytotoxicity of NaDES-derived lipid extracts

The CTB assay (Figure 18) confirmed the absence of cytotoxic effects for all NaDES-derived lipid extracts in both epithelial cell models. In HCEC-1CT, cell viability remained tightly clustered around the DMSO control (97–106 %, SD < 6 %), and Dunnett's post-hoc test showed no statistically significant deviations ($p > 0.05$). Similarly, all treatments in HCT116 maintained high metabolic activity, with mean values between 92 and 109 %. Two conditions exhibited small but statistically significant shifts relative to the control: a slight increase for the C8:C12-based lipid extract at a tested concentration of $100 \mu\text{g mL}^{-1}$ (+7 %) and a mild decrease for the Me:Th-based lipid extract at $100 \mu\text{g mL}^{-1}$ (−8 %). However, both values remained high overall, suggesting that the statistically detectable differences are unlikely to translate into substantial biological effects. Thus, the absence of substantial, concentration-dependent decreases in viability in both cell lines suggests that the NaDES-derived lipid extracts do not exert cytotoxic effects at concentrations up to $100 \mu\text{g mL}^{-1}$.

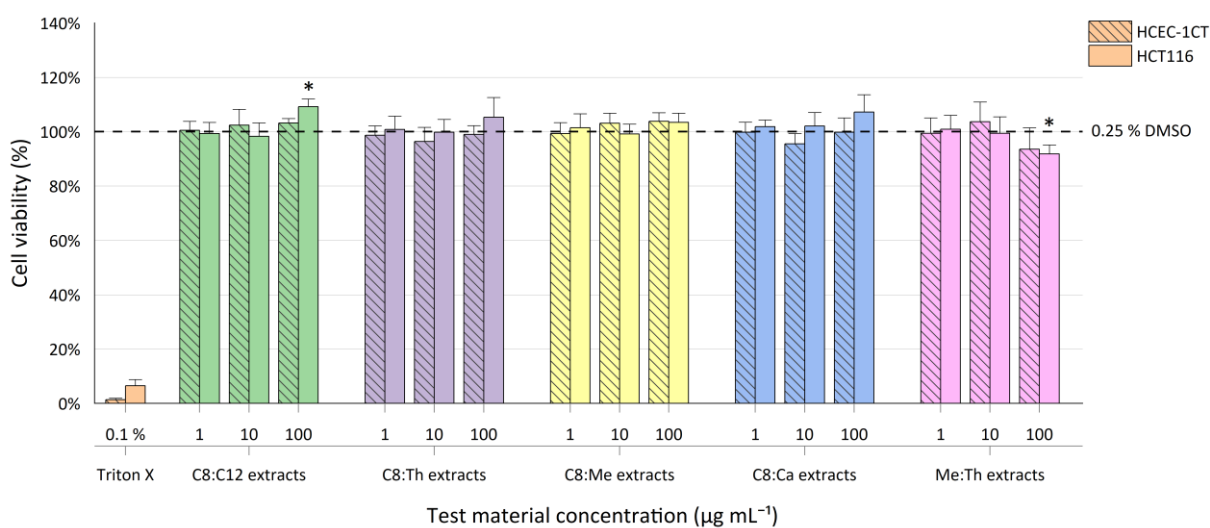


Figure 18: CTB assay – Impact of NaDES-derived lipid extracts on viability of HCEC-1CT and HCT116 cells. Cells were incubated with different concentrations (1, 10, $100 \mu\text{g mL}^{-1}$) of five NaDES-derived lipid extracts for 24 h. Viability was measured by CTB and normalized to the 0.25 % DMSO control;

Triton X-100 (0.1 %) served as positive control. Statistical significance was assessed by one-way ANOVA with Dunnett's test (* $p < 0.05$).

The SRB assay (Figure 19) confirmed the generally non-cytotoxic character of all NaDES-derived lipid extracts. In HCEC-1CT, mean viability values ranged between 87 % and 120 %, with several samples, particularly the C8:C12 and C8:Ca-based lipid extracts, exceeding 100 % of the control. However, these upward tendencies were not statistically significant. HCEC-1CT showed no cytotoxicity at any concentration. In HCT116, viability values were more variable (64–106 %), and a concentration-dependent decline became evident for the Me:Th-based lipid extract (viability: 63.6 ± 7.9 % at $100 \mu\text{g mL}^{-1}$). Dunnett's post-hoc test identified this as the only statistically significant deviation from the DMSO control. The remaining extracts maintained mean viabilities above 80 %, suggesting the absence of relevant cytotoxic effects. Overall, both cell lines displayed high tolerance toward the NaDES-derived lipid extracts, with only a mild effect observed with Me:Th-based lipid extracts at $100 \mu\text{g mL}^{-1}$.

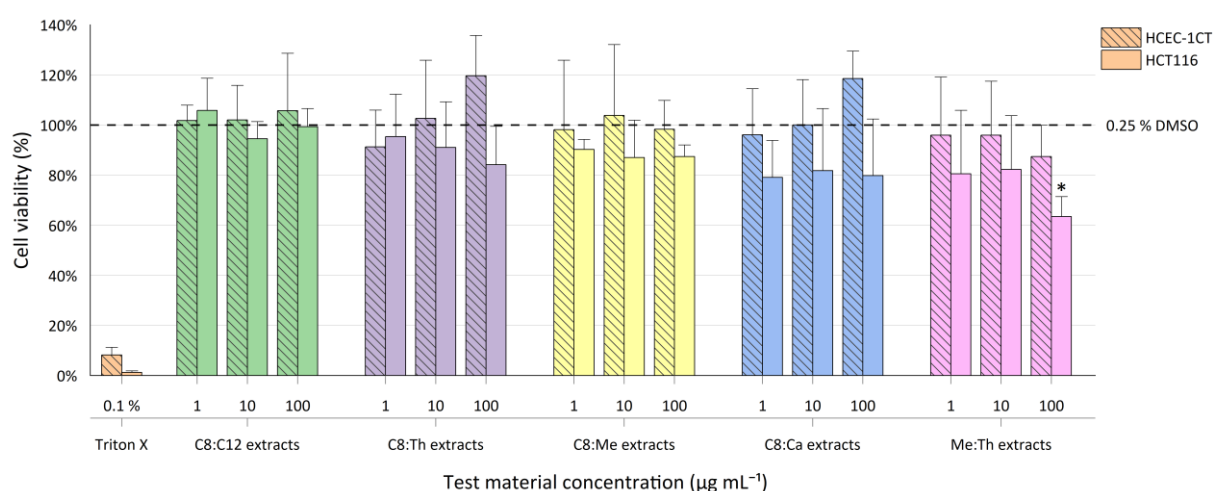


Figure 19: SRB assay – Impact of NaDES-derived lipid extracts on viability of HCEC-1CT and HCT116 cells. Cells were incubated with different concentrations (1, 10, $100 \mu\text{g mL}^{-1}$) of five NaDES-derived lipid extracts for 24 h. Viability was measured by SRB and normalized to the 0.25 % DMSO control; Triton X-100 (0.1 %) served as positive control. Statistical significance was assessed by one-way ANOVA with Dunnett's test (* $p < 0.05$).

The CTB assay results of the Soxhlet-extracted lipids and the commercial reference oil (Figure 20) demonstrated uniformly high cell viability for both samples. In HCEC-1CT, Soxhlet extracts yielded values between 97 % and 101 %, showing no significant deviation from the DMSO control. The commercial oil, however, produced a moderate yet statistically significant increase in viability at 10 and $100 \mu\text{g}$

mL⁻¹ (+9 % and +14 %, respectively), suggesting a mild proliferative or metabolic activation effect rather than cytotoxicity. In HCT116, neither the Soxhlet extracts nor the commercial oil induced significant changes, and all mean values remained within 97–106 % of the control. The consistent trend toward slightly elevated viability at higher concentrations, especially in the epithelial HCEC-1CT line, indicates that both lipid matrices are well tolerated and may even stimulate cellular metabolism under non-toxic conditions.

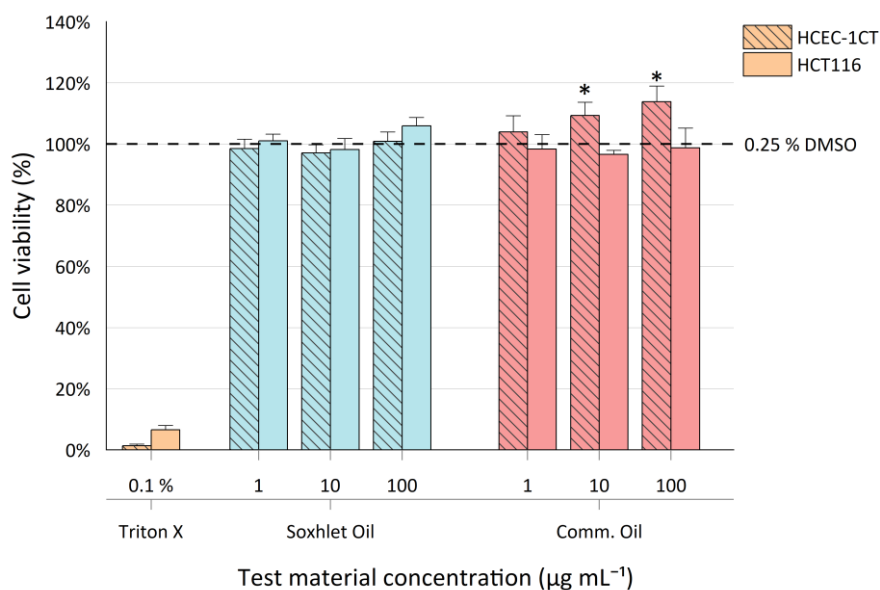


Figure 20: CTB assay – Impact of Soxhlet extracted and commercial oil on viability of HCEC-1CT and HCT116 cells. Cells were incubated with different concentrations (1, 10, 100 µg mL⁻¹) of Soxhlet and Commercial oil for 24 h. Viability was measured by CTB and normalized to the 0.25 % DMSO control; Triton X-100 (0.1 %) served as positive control. Statistical significance was assessed by one-way ANOVA with Dunnett's test (* $p < 0.05$).

The SRB assay results of Soxhlet-extracted and commercial oil (Figure 21) confirmed the absence of cytotoxic effects for both samples. In HCEC-1CT, cell viability remained between 93 % and 112 % for the Soxhlet extracts and between 90 % and 100 % for the commercial oil, with none of the treatments showing a statistically significant difference compared to DMSO. Similarly, HCT116 cells displayed stable viability values across all concentrations (85–105 %), without any significant deviations. As in the CTB assay, both cell lines exhibited a consistent upward trend in viability at higher concentrations, particularly for the Soxhlet extracts, indicating a potential mild stimulation of cellular proliferation or protein synthesis rather than toxicity. Across all treatments, mean viability values remained above 85 %, suggesting the absence of relevant cytotoxic effects up to a tested concentration of 100 µg mL⁻¹.

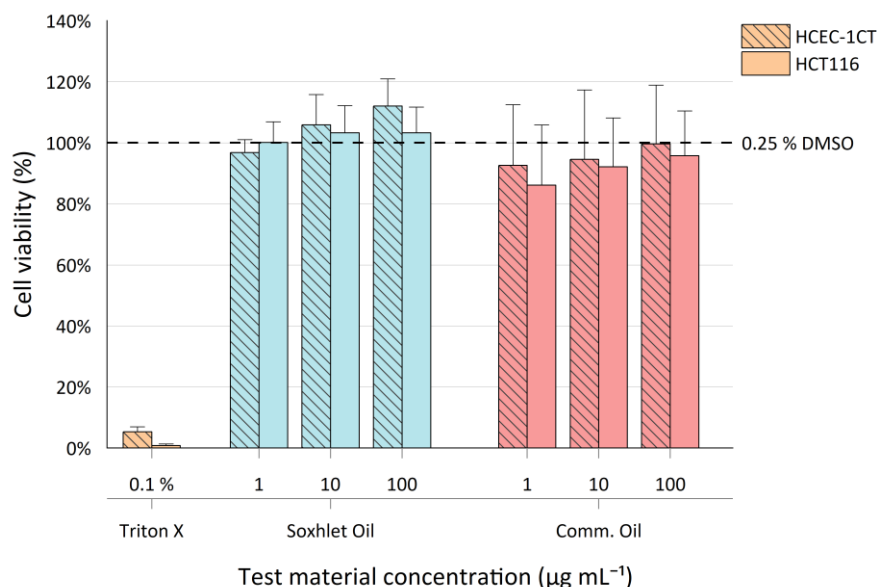


Figure 21: SRB assay – Impact of Soxhlet extracted and commercial oil on viability of HCEC-1CT and HCT116 cells. Cells were incubated with different concentrations (1, 10, 100 µg mL⁻¹) of Soxhlet and Commercial oil for 24 h. Viability was measured by SRB and normalized to the 0.25 % DMSO control; Triton X-100 (0.1 %) served as positive control. Statistical significance was assessed by one-way ANOVA with Dunnett's test (* $p < 0.05$).

5.3.2 Cytotoxicity of pure NaDES mixtures

After evaluating the NaDES-derived lipid extracts, the impact on cell viability of the pure NaDES mixtures was examined to assess their intrinsic cytotoxic potential. This comparison allows to distinguish whether the small viability shifts seen for the extracts originated from the pure NaDES mixtures or the lipids extracts.

The CTB assay of the pure NaDES mixtures (Figure 22) revealed high overall cell viability for all NaDES mixtures, confirming their general non-toxicity within the tested concentration range. In HCEC-1CT, most samples remained close to the DMSO control, with mean values between 87 % and 109 %. Statistically significant deviations were observed for the pure C8:C12 NaDES at a test concentration of 100 µg mL⁻¹ (-12 %) and C8:Ca at 10 µg mL⁻¹ (+7 %). In contrast, HCT116 cells showed no significant differences across any treatment, with mean values between 92 % and 104 %. Overall, all NaDES remained non-cytotoxic up to tested concentrations of 100 µg mL⁻¹.

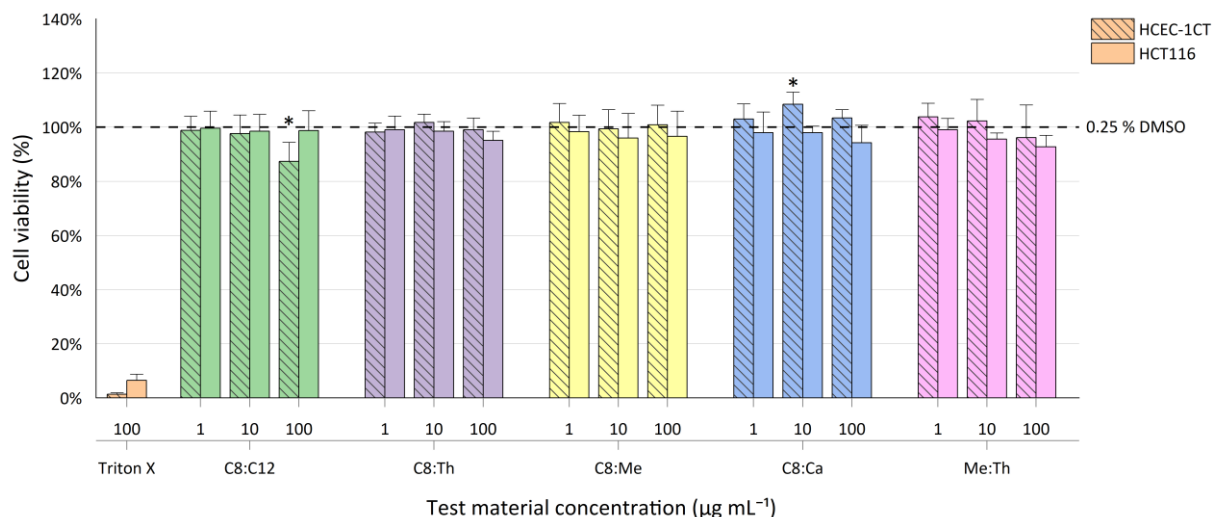


Figure 22: CTB assay – Impact of pure NaDES mixtures on viability of HCEC-1CT and HCT116 cells. Cells were incubated with different concentrations (1, 10, 100 µg mL⁻¹) of pure NaDES mixtures for 24 h. Viability was measured by CTB and normalized to the 0.25 % DMSO control; Triton X-100 (0.1 %) served as positive control. Statistical significance was assessed by one-way ANOVA with Dunnett's test (* $p < 0.05$).

The SRB assay of the NaDES mixtures (Figure 23) showed a moderate reduction in cell viability for some NaDES mixtures at higher concentrations, yet no statistically significant differences were detected in HCEC-1CT cells with mean viability values ranging from 79 % to 96 %. In HCT116, most of the treatments resulted in a cell viability above 85 %, except for the Me:Th NaDES at a tested concentration of 100 µg mL⁻¹, which induced a statistically significant reduction (viability: ~73 %). Overall, the SRB results confirm that all NaDES systems are non-cytotoxic up to 100 µg mL⁻¹, with only the Me:Th NaDES at 100 µg mL⁻¹ showing a mild cytotoxic effect.

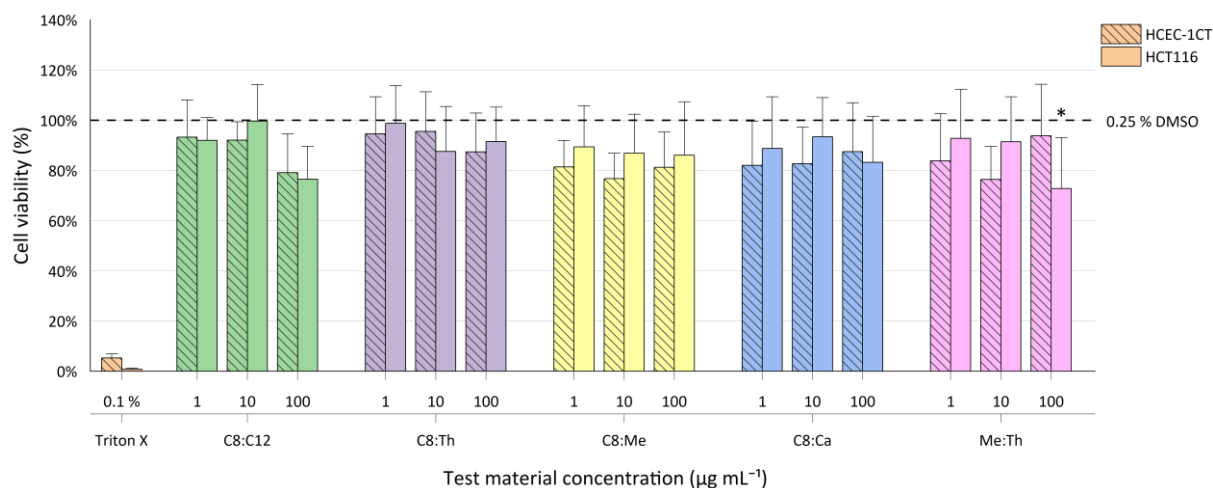


Figure 23: SRB assay – Impact of pure NaDES mixtures on viability of HCEC-1CT and HCT116 cells. Cells were incubated with different concentrations (1, 10, 100 µg mL⁻¹) of pure NaDES mixtures for 24 h. Viability was measured by SRB and normalized to the 0.25 % DMSO control; Triton X-100 (0.1 %) served as positive control. Statistical significance was assessed by one-way ANOVA with Dunnett's test (* $p < 0.05$).

Taken together, the data suggest that the tested NaDES systems impose only minimal cellular stress at concentrations up to 100 µg mL⁻¹, with no indication of pronounced cytotoxicity in either assay or cell line.

5.3.3 Cytotoxicity of single NaDES components

To fully examine the potential of the single NaDES components to exert cytotoxic effects, further CTB and SRB assays were performed.

The CTB assay of the single NaDES components (Figure 24) revealed distinct, concentration-dependent effects among the individual NaDES components. In HCEC-1CT, several compounds caused statistically significant reductions in metabolic activity, with mild to pronounced cytotoxic responses for caprylic acid (100 µg mL⁻¹), lauric acid (25, 100 µg mL⁻¹), carvacrol (25, 100 µg mL⁻¹), thymol (25, 75, 100 µg mL⁻¹) and Jeffamine D-230 (25 µg mL⁻¹) with viability reductions of 85-92 % at concentrations of 25 µg mL⁻¹ and reductions down to 16 % for concentrations of 100 µg mL⁻¹. In HCT116, a similar pattern was observed, though the overall sensitivity was slightly lower. Statistically significant reductions occurred for caprylic acid (100 µg mL⁻¹), lauric acid (100 µg mL⁻¹), carvacrol (100 µg mL⁻¹), thymol (75, 100 µg mL⁻¹) and Jeffamine (100 µg mL⁻¹) with viability reductions of 69-88 %.

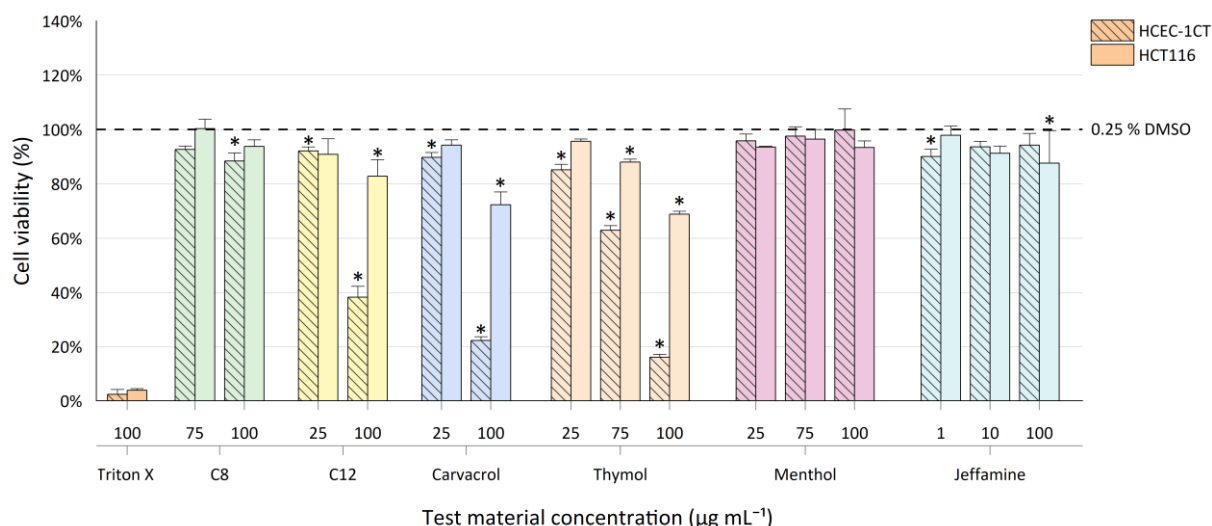


Figure 24: CTB assay – Impact of single NaDES components and Jeffamine on viability of HCEC-1CT and HCT116 cells. Cells were incubated with different concentrations of single NaDES components (25, 75, 100 μg mL⁻¹) or Jeffamine solution (1, 10, 100 μg mL⁻¹) for 24 h. Viability was measured by CTB and normalized to the 0.25 % DMSO control; Triton X-100 (0.1 %) served as positive control. Statistical significance was assessed by one-way ANOVA with Dunnett's test (* p < 0.05).

The SRB assay of the single NaDES components (Figure 25) revealed several statistically significant reductions in cell viability, particularly at higher concentrations of specific NaDES components. In HCEC-1CT, the most pronounced effects were observed for lauric acid (100 μg mL⁻¹), thymol (100 μg mL⁻¹), carvacrol (100 μg mL⁻¹) and menthol (75 μg mL⁻¹) each showing a reduction in cell viability down to 25-79 %. These results indicate strong cytotoxic potential associated with phenolic compounds (thymol, carvacrol) and medium-chain saturated fatty acids (C12). In HCT116, significant reductions in viability were observed for lauric acid (100 μg mL⁻¹), carvacrol (100 μg mL⁻¹), thymol (75 and 100 μg mL⁻¹), menthol (100 μg mL⁻¹) and Jeffamine D-230 (100 μg mL⁻¹) with reductions of 53-85 %. These findings mirror those in HCEC-1CT, emphasizing the cytotoxic potential of phenolic structures and medium-chain fatty acids across both epithelial and colorectal cell models.

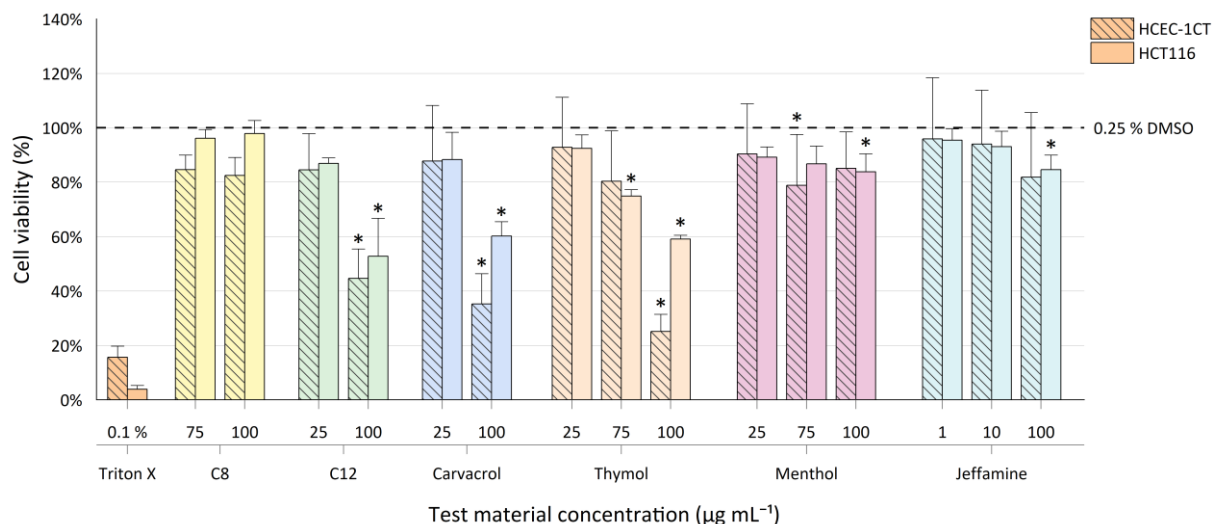


Figure 25: SRB assay – Impact of single NaDES components and Jeffamine on viability of HCEC-1CT and HCT116 cells. Cells were incubated with different concentrations of single NaDES components (25, 75, 100 µg mL⁻¹) or Jeffamine solution (1, 10, 100 µg mL⁻¹) for 24 h. Viability was measured by SRB and normalized to the 0.25 % DMSO control; Triton X-100 (0.1 %) served as positive control. Statistical significance was assessed by one-way ANOVA with Dunnett’s test (* p < 0.05).

Overall, both cell lines exhibited the highest sensitivity toward phenolic compounds (thymol, carvacrol), followed by a moderate response to medium-chain fatty acids (C12). In contrast, short-chain fatty acids (C8), menthol, and the amine-based compound Jeffamine were generally well tolerated. These findings indicate that cytotoxicity within the tested NaDES systems primarily originates from functional groups with high membrane-disruptive or protein-denaturing potential, whereas the majority of non-phenolic single components remain non-toxic under moderate exposure conditions.

5.3.4 Integrated interpretation and cross-comparison

To facilitate systematic interpretation, each NaDES system was evaluated using the same logic: cytotoxicity of the extract, cytotoxicity of the pure NaDES mixtures, cytotoxicity of single components, and correlation with the quantified residual NaDES content in the extract calculated in Section 4.2.4. This allows a direct comparison between systems and makes it possible to identify whether cytotoxic responses originate from the NaDES mixture, its individual components, or constituents present in the lipid extract. It is important to note that, as demonstrated in Section 5.1.14, caprylic acid was fully removed during recovery, whereas varying residual levels of the respective partner component (C12,

thymol, menthol, carvacrol) remained in the extracts. Consequently, any extract-related cytotoxic effects discussed in the following sections can be attributed exclusively to the non-C8 NaDES constituent.

The C8:C12 extract showed no indications of cytotoxicity in either cell line, with CTB and SRB viabilities consistently remaining above 95 % across all concentrations. In contrast, the pure C8:C12 NaDES exhibited a moderate viability decrease at $100\text{ }\mu\text{g mL}^{-1}$, particularly in SRB (79 % in HCEC-1CT; 77 % in HCT116), indicating primarily effects at high concentrations. Component testing revealed that these effects originate almost exclusively from C12, which substantially reduced viability at $100\text{ }\mu\text{g mL}^{-1}$ in both assays, while C8 remained largely non-toxic. Importantly, the extract did not reproduce these effects, indicating that residual C12 levels are far below cytotoxic thresholds. The absence of cytotoxic effects in the C8:C12 extract is consistent with its composition, which contained 16 % of C12 and no detectable C8. At the applied extract concentrations, this corresponded to residual C12 levels too low to induce measurable toxicity.

The C8:Th extract showed no indications of cytotoxicity in either cell line, with CTB and SRB viabilities remaining stable across all concentrations. The pure C8:Th NaDES combinations was likewise well tolerated and did not reproduce the toxicity patterns observed for the isolated components. In the single-component assays, cytotoxic effects were attributable exclusively to thymol. In HCEC-1CT, thymol caused significant reductions in metabolic activity at 75 and $100\text{ }\mu\text{g mL}^{-1}$ (CTB) and at $100\text{ }\mu\text{g mL}^{-1}$ in the SRB assay. In HCT116, thymol induced viability loss in CTB starting at $75\text{ }\mu\text{g mL}^{-1}$ and in SRB at both 75 and $100\text{ }\mu\text{g mL}^{-1}$. Caprylic acid showed only a moderate decrease in HCEC-1CT (CTB, $100\text{ }\mu\text{g mL}^{-1}$) and remained non-toxic under all other conditions. The absence of toxicity in the C8:Th extract is fully consistent with its chemical composition: the extract contained 51 % thymol but no detectable C8. Consequently, even at the highest extract concentration, the effective thymol level in the assay ($\sim 51\text{ }\mu\text{g mL}^{-1}$) remained well below the threshold at which thymol caused toxicity in the single-component tests.

The C8:Me extract was well tolerated in both cell models, with no reductions in CTB or SRB viability across the tested concentration range. The pure C8:Me NaDES likewise showed no cytotoxic responses, indicating that neither the solvent system nor the recovered extract induces measurable metabolic or structural impairment under the applied conditions. In contrast, the single-component assays revealed mild, concentration-dependent effects for menthol: in HCEC-1CT, a significant decrease in SRB viability occurred at $75\text{ }\mu\text{g mL}^{-1}$, whereas in HCT116, SRB viability was reduced at $100\text{ }\mu\text{g mL}^{-1}$. Caprylic acid again produced only a modest viability loss at $100\text{ }\mu\text{g mL}^{-1}$ (CTB, HCEC-1CT), with no

broader cytotoxicity across assays or cell lines. The benign toxicity profile of the C8:Me extract aligns with its chemical composition. Menthol constituted 71 % of the extract, while no residual C8 was detected. Even at the highest extract concentration, the effective menthol level ($\sim 71 \mu\text{g mL}^{-1}$) remained below the threshold at which toxicity occurred.

The C8:Ca extract showed no cytotoxic effects in either assay or cell line, and viability consistently remained within the physiological range. The pure C8:Ca NaDES likewise elicited no relevant toxicity, with the exception of a slight increase in CTB metabolic activity in HCEC-1CT at $10 \mu\text{g mL}^{-1}$, which does not indicate adverse effects. In contrast, the single-component assays revealed clear concentration-dependent responses for carvacrol: both CTB and SRB showed significant viability reductions at $100 \mu\text{g mL}^{-1}$ in HCEC-1CT and HCT116. Caprylic acid again displayed only a minor viability decrease at $100 \mu\text{g mL}^{-1}$ in HCEC-1CT (CTB), with no broader effects. The non-toxic behavior of the C8:Ca extract reflects its composition: carvacrol accounted for 46 % of the extract, while C8 was fully removed. At the maximal extract concentration, this corresponds to an effective carvacrol level of $\sim 46 \mu\text{g mL}^{-1}$ in the assay and was therefore below the concentrations at which isolated carvacrol showed toxicity.

The Me:Th extract showed a distinct behavior compared to the other systems. In HCT116, a moderate reduction in CTB viability occurred at $100 \mu\text{g mL}^{-1}$ ($\sim 8 \%$), accompanied by a clear decrease in SRB viability at the same concentration, while no reductions were observed in HCEC-1CT. The pure Me:Th NaDES elicited an almost identical response, causing a viability decrease in HCT116 (SRB, $100 \mu\text{g mL}^{-1}$), indicating that both extract and NaDES share the same underlying cytotoxicity profile. The Me:Th extracts and the pure Me:Th NaDES exhibit a similar toxicity profile, reflecting the fact that no recovery strategy successfully separated the NaDES components from the lipid fraction. As a result, the extract and the pure NaDES shared an almost identical chemical composition, with menthol and thymol present in a 1:1 molar ratio and only a minor fraction of lipids added in the extract. The single-component assays support this interpretation. As observed previously, thymol showed pronounced concentration-dependent cytotoxicity in both cell models, with reductions at 75 and $100 \mu\text{g mL}^{-1}$ (CTB and SRB), while menthol induced SRB toxicity only at higher concentrations ($75 \mu\text{g mL}^{-1}$ in HCEC-1CT; $100 \mu\text{g mL}^{-1}$ in HCT116). Since the Me:Th extract contains both components in roughly equal proportions and with no removal during processing, the effective concentrations of thymol and menthol at the highest extract dose fall precisely within the range in which these molecules exerted cytotoxic effects when tested individually. Therefore, the extract inherently reproduces the toxicity profile of the pure NaDES, and the observed reductions in viability can be directly attributed to the combined action of menthol and thymol at concentrations exceeding those tolerated in the single-component assays.

Across all tested systems, the cytotoxicity evaluation revealed a consistent pattern: none of the recovered lipid extracts exhibited biologically relevant toxicity in either epithelial cell line. This demonstrates that the extraction procedures successfully yielded non-cytotoxic lipid fractions, even when single NaDES components showed cytotoxic effects at higher concentrations. The only exception was Me:Th, where no successful recovery was achieved, and the extract therefore mirrored the cytotoxicity of the pure NaDES mixture.

Taken together, these findings show that NaDES-related cytotoxicity is determined by the single components and that effective removal of NaDES during recovery is the critical determinant of extract safety. When the NaDES components are removed efficiently, the resulting extracts are well tolerated *in vitro*. It should also be noted that the concentrations required to induce cytotoxicity in isolated components exceed realistic exposure levels by several orders of magnitude, indicating that any residual NaDES traces in food-grade extracts would be below toxicologically relevant thresholds.

6 Conclusion

This study demonstrates that hydrophobic and fatty-acid-based NaDES represent effective and more sustainable alternatives to conventional hexane-based extraction of *Schizochytrium sp.* lipids. Optimized probe-assisted ultrasound extraction enabled rapid and efficient lipid recovery, achieving fatty acid yields comparable to or exceeding Soxhlet extraction while significantly reducing processing time and solvent consumption. A key finding of this work is that fatty-acid-based NaDES, in combination with Jeffamine-induced polarity switching, allow effective separation of the lipid extract from the solvent phase. Among the investigated systems, C8:C12 showed the most favorable performance, combining high extract purity with minimal solvent carryover, whereas terpene-containing systems exhibited incomplete phase separation due to the absence of fully switchable components. Importantly, cytotoxicity assessment confirmed that both NaDES and NaDES-derived extracts are well tolerated in HCEC-1CT and HCT116 cells at concentrations up to 100 $\mu\text{g mL}^{-1}$, indicating no additional *in vitro* safety concerns compared to conventionally extracted oils. These findings highlight that the incorporation of protonatable components is a critical design principle for NaDES intended for reversible extraction processes. Overall, this work provides a coherent proof-of-concept that NaDES-based extraction can simultaneously address efficiency, recoverability and safety, supporting their potential application in the sustainable production of omega-3-rich lipids from marine microorganisms.

7 References

- [1] G. Breuer *et al.*, "Analysis of Fatty Acid Content and Composition in Microalgae," *J. Vis. Exp. JoVE*, no. 80, p. 50628, Oct. 2013, doi: 10.3791/50628.
- [2] E. Ryckebosch, K. Muylaert, and I. Foubert, "Optimization of an Analytical Procedure for Extraction of Lipids from Microalgae," *J. Am. Oil Chem. Soc.*, vol. 89, no. 2, pp. 189–198, Feb. 2012, doi: 10.1007/s11746-011-1903-z.
- [3] C. Da Porto, E. Porretto, and D. Decorti, "Comparison of ultrasound-assisted extraction with conventional extraction methods of oil and polyphenols from grape (*Vitis vinifera* L.) seeds," *Ultrason. Sonochem.*, vol. 20, no. 4, pp. 1076–1080, Jul. 2013, doi: 10.1016/j.ultsonch.2012.12.002.
- [4] C. Florindo, L. C. Branco, and I. M. Marrucho, "Quest for Green-Solvent Design: From Hydrophilic to Hydrophobic (Deep) Eutectic Solvents," *ChemSusChem*, vol. 12, no. 8, pp. 1549–1559, 2019, doi: 10.1002/cssc.201900147.
- [5] S. Mehariya, F. Fratini, R. Lavecchia, and A. Zuurro, "Green extraction of value-added compounds from microalgae: A short review on natural deep eutectic solvents (NaDES) and related pre-treatments," *J. Environ. Chem. Eng.*, vol. 9, no. 5, p. 105989, Oct. 2021, doi: 10.1016/j.jece.2021.105989.
- [6] M. Hannon, J. Gimpel, M. Tran, B. Rasala, and S. Mayfield, "Biofuels from algae: challenges and potential," *Biofuels*, vol. 1, no. 5, pp. 763–784, Sep. 2010, doi: 10.4155/bfs.10.44.
- [7] "High-value products from microalgae—Their development and commercialisation | Request PDF," *ResearchGate*, Aug. 2025, doi: 10.1007/s10811-013-9983-9.
- [8] M. Leal Nörnberg *et al.*, "Current Status of Microalgae-based Food Products: Future Trends of Functional Ingredients," *Recent Pat. Biotechnol.*, Aug. 2025, doi: 10.2174/0118722083380758250802021828.
- [9] Q. Hu *et al.*, "Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances," *Plant J. Cell Mol. Biol.*, vol. 54, no. 4, pp. 621–639, May 2008, doi: 10.1111/j.1365-313X.2008.03492.x.

- [10] D. B. Jump, C. M. Depner, and S. Tripathy, "Omega-3 fatty acid supplementation and cardiovascular disease: Thematic Review Series: New Lipid and Lipoprotein Targets for the Treatment of Cardiometabolic Diseases," *J. Lipid Res.*, vol. 53, no. 12, pp. 2525–2545, Dec. 2012, doi: 10.1194/jlr.R027904.
- [11] M. L. Menegazzo and G. G. Fonseca, "Biomass recovery and lipid extraction processes for microalgae biofuels production: A review," *Renew. Sustain. Energy Rev.*, vol. 107, pp. 87–107, Jun. 2019, doi: 10.1016/j.rser.2019.01.064.
- [12] T. M. Mata, A. A. Martins, and Nidia. S. Caetano, "Microalgae for biodiesel production and other applications: A review," *Renew. Sustain. Energy Rev.*, vol. 14, no. 1, pp. 217–232, Jan. 2010, doi: 10.1016/j.rser.2009.07.020.
- [13] C. Florindo, L. Romero, I. Rintoul, L. C. Branco, and I. M. Marrucho, "From Phase Change Materials to Green Solvents: Hydrophobic Low Viscous Fatty Acid–Based Deep Eutectic Solvents," *ACS Sustain. Chem. Eng.*, vol. 6, no. 3, pp. 3888–3895, Mar. 2018, doi: 10.1021/acssuschemeng.7b04235.
- [14] R. Yokoyama and D. Honda, "Taxonomic rearrangement of the genus *Schizochytrium* sensu lato based on morphology, chemotaxonomic characteristics, and 18S rRNA gene phylogeny (Thraustochytriaceae, Labyrinthulomycetes): emendation for *Schizochytrium* and erection of *Aurantiochytrium* and *Oblongichytrium* gen. nov.," *Mycoscience*, vol. 48, no. 4, pp. 199–211, 2007, doi: 10.1007/S10267-006-0362-0.
- [15] Z. Cohen and C. Ratledge, Eds., *Single Cell Oils: Microbial and Algal Oils*. New York: AOCS Publishing, 2005. doi: 10.4324/9781003040101.
- [16] European Commission, "Commission Implementing Regulation (EU) 2022/1365 of 4 August 2022." Official Journal of the European Union, 2022. [Online]. Available: <https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32022R1365>
- [17] L. Zhu, X. Zhang, L. Ji, X. Song, and C. Kuang, "Changes of lipid content and fatty acid composition of *Schizochytrium limacinum* in response to different temperatures and salinities," *Process Biochem.*, vol. 42, no. 2, pp. 210–214, Feb. 2007, doi: 10.1016/j.procbio.2006.08.002.

- [18] G. Berzal, M. P. García García, and F. J. Señorans Rodríguez, "Integrated Process for Schizochytrium Oil Extraction, Enzymatic Modification of Lipids and Concentration of DHA Fatty Acid Esters Using Alternative Methodologies," Mar. 2024, doi: 10.3390/md22040146.
- [19] N. Kujawska, S. Talbierz, M. Dębowski, J. Kazimierowicz, and M. Zieliński, "Cultivation Method Effect on Schizochytrium sp. Biomass Growth and Docosahexaenoic Acid (DHA) Production with the Use of Waste Glycerol as a Source of Organic Carbon," *Energies*, vol. 14, no. 10, p. 2952, Jan. 2021, doi: 10.3390/en14102952.
- [20] L. A. Horrocks and Y. K. Yeo, "Health benefits of docosahexaenoic acid (DHA)," *Pharmacol. Res.*, vol. 40, no. 3, pp. 211–225, Sep. 1999, doi: 10.1006/phrs.1999.0495.
- [21] M. Rodríguez-España, L. G. Mendoza-Sánchez, P. Magallón-Servín, M. A. Salgado-Cervantes, A. A. Acosta-Osorio, and H. S. García, "Supercritical fluid extraction of lipids rich in DHA from *Schizochytrium* sp.," *J. Supercrit. Fluids*, vol. 179, p. 105391, Jan. 2022, doi: 10.1016/j.supflu.2021.105391.
- [22] A. P. Abbott, D. Boothby, G. Capper, D. L. Davies, and R. K. Rasheed, "Deep Eutectic Solvents Formed between Choline Chloride and Carboxylic Acids: Versatile Alternatives to Ionic Liquids," *J. Am. Chem. Soc.*, vol. 126, no. 29, pp. 9142–9147, Jul. 2004, doi: 10.1021/ja048266j.
- [23] A. Mišan *et al.*, "The perspectives of natural deep eutectic solvents in agri-food sector," *Crit. Rev. Food Sci. Nutr.*, vol. 60, no. 15, pp. 2564–2592, Aug. 2020, doi: 10.1080/10408398.2019.1650717.
- [24] Y. Liu, J. B. Friesen, J. B. McAlpine, D. C. Lankin, S.-N. Chen, and G. F. Pauli, "Natural Deep Eutectic Solvents: Properties, Applications, and Perspectives," *J. Nat. Prod.*, vol. 81, no. 3, pp. 679–690, Mar. 2018, doi: 10.1021/acs.jnatprod.7b00945.
- [25] L. Wils *et al.*, "Natural deep eutectic solvent formulations for spirulina: Preparation, intensification, and skin impact," *Algal Res.*, vol. 56, p. 102317, Jun. 2021, doi: 10.1016/j.algal.2021.102317.
- [26] D. O. Abranches, M. A. R. Martins, L. P. Silva, N. Schaeffer, S. P. Pinho, and J. A. P. Coutinho, "Phenolic hydrogen bond donors in the formation of non-ionic deep eutectic solvents: the quest for type V DES," *Chem. Commun.*, vol. 55, no. 69, pp. 10253–10256, Aug. 2019, doi: 10.1039/C9CC04846D.

- [27] D. J. G. P. van Osch, L. F. Zubeir, A. van den Bruinhorst, M. A. A. Rocha, and M. C. Kroon, "Hydrophobic deep eutectic solvents as water-immiscible extractants," *Green Chem.*, vol. 17, no. 9, pp. 4518–4521, Sep. 2015, doi: 10.1039/C5GC01451D.
- [28] T. Topal *et al.*, "Hydrophobic natural deep eutectic solvents for marine lipid extraction," *J. Am. Oil Chem. Soc.*, vol. 101, no. 3, pp. 361–367, 2024, doi: 10.1002/aocs.12757.
- [29] N. Rodriguez Rodriguez, A. van den Bruinhorst, L. J. B. M. Kollau, M. C. Kroon, and K. Binnemans, "Degradation of Deep-Eutectic Solvents Based on Choline Chloride and Carboxylic Acids," *ACS Sustain. Chem. Eng.*, vol. 7, no. 13, pp. 11521–11528, Jul. 2019, doi: 10.1021/acssuschemeng.9b01378.
- [30] X. Zheng *et al.*, "An overview of hydrophobic deep eutectic solvents driven liquid-phase extraction: Applications and prospects," *J. Chromatogr. A*, vol. 1748, p. 465824, May 2025, doi: 10.1016/j.chroma.2025.465824.
- [31] Q. Zhang, K. D. O. Vigier, S. Royer, and F. Jérôme, "Deep eutectic solvents: syntheses, properties and applications," *Chem. Soc. Rev.*, vol. 41, no. 21, pp. 7108–7146, Oct. 2012, doi: 10.1039/C2CS35178A.
- [32] M. Cherniakova, V. Varchenko, and K. Belikov, "Menthol-Based (Deep) Eutectic Solvents: A Review on Properties and Application in Extraction," *Chem. Rec.*, vol. 24, no. 2, p. e202300267, 2024, doi: 10.1002/tcr.202300267.
- [33] J. Cao and E. Su, "Hydrophobic deep eutectic solvents: the new generation of green solvents for diversified and colorful applications in green chemistry," *J. Clean. Prod.*, vol. 314, p. 127965, Sep. 2021, doi: 10.1016/j.jclepro.2021.127965.
- [34] D. S. Freitas, A. Cavaco-Paulo, and C. Silva, "Enhancing insights into the phenomena of deep eutectic solvents," *Sustain. Mater. Technol.*, vol. 41, p. e01039, Sep. 2024, doi: 10.1016/j.susmat.2024.e01039.
- [35] C. Cai, Y. Wang, W. Yu, C. Wang, F. Li, and Z. Tan, "Temperature-responsive deep eutectic solvents as green and recyclable media for the efficient extraction of polysaccharides from *Ganoderma lucidum*," *J. Clean. Prod.*, vol. 274, p. 123047, Nov. 2020, doi: 10.1016/j.jclepro.2020.123047.

- [36] L. Sportiello *et al.*, "Hydrophobic deep eutectic solvents in the food sector: Focus on their use for the extraction of bioactive compounds," *Food Chem.*, vol. 405, p. 134703, Mar. 2023, doi: 10.1016/j.foodchem.2022.134703.
- [37] A. Kyriakoudi *et al.*, "Coupling deep eutectic solvents with innovative extraction techniques towards plant derived bioactive compositions," *RSC Sustain.*, vol. 2, no. 6, pp. 1675–1691, Jun. 2024, doi: 10.1039/D4SU00111G.
- [38] L. Yu *et al.*, "Recovery and post-treatment processes for ionic liquids and deep eutectic solvents," *J. Mol. Liq.*, vol. 402, p. 124767, May 2024, doi: 10.1016/j.molliq.2024.124767.
- [39] C. Lo, R. H. Wijffels, and M. H. M. Eppink, "Lipid recovery from deep eutectic solvents by polar antisolvents," *Food Bioprod. Process.*, vol. 143, pp. 21–27, Jan. 2024, doi: 10.1016/j.fbp.2023.10.003.
- [40] S. Della Posta, V. Gallo, A. Gentili, and C. Fanali, "Strategies for the recovery of bioactive molecules from deep eutectic solvents extracts," *TrAC Trends Anal. Chem.*, vol. 157, p. 116798, Dec. 2022, doi: 10.1016/j.trac.2022.116798.
- [41] S. S. Patil, A. Pathak, and V. K. Rathod, "Optimization and kinetic study of ultrasound assisted deep eutectic solvent based extraction: A greener route for extraction of curcuminoids from *Curcuma longa*," *Ultrason. Sonochem.*, vol. 70, p. 105267, Jan. 2021, doi: 10.1016/j.ultsonch.2020.105267.
- [42] Q. Yu *et al.*, "Catalytic conversion of herbal residue carbohydrates to furanic derivatives in a deep eutectic solvent accompanied by dissolution and recrystallisation of choline chloride," *Cellulose*, vol. 26, no. 15, pp. 8263–8277, Oct. 2019, doi: 10.1007/s10570-019-02372-6.
- [43] L. Marinaccio *et al.*, "Ultrasound assisted deep eutectic solvent-based extraction of Montepulciano d' Abruzzo grape seeds for the recovery of the grape seed oil and its biological evaluation," *Food Chem. X*, vol. 26, p. 102273, Feb. 2025, doi: 10.1016/j.fochx.2025.102273.
- [44] G. Sed, A. Cicci, P. G. Jessop, and M. Bravi, "A novel switchable-hydrophilicity, natural deep eutectic solvent (NaDES)-based system for bio-safe biorefinery," *RSC Adv.*, vol. 8, no. 65, pp. 37092–37097, Nov. 2018, doi: 10.1039/C8RA08536F.

- [45] E. Ryckebosch *et al.*, "Influence of extraction solvent system on the extractability of lipid components from the biomass of *Nannochloropsis gaditana*," *J. Appl. Phycol.*, vol. 26, no. 3, pp. 1501–1510, Jun. 2014, doi: 10.1007/s10811-013-0189-y.
- [46] F. Soxhlet, "Die gewichtsanalytische Bestimmung des MilCHFettes," *Dinglers Polytech. J.*, no. 232, pp. 461–465., 1879.
- [47] J. Folch, M. Lees, and G. H. S. Stanley, "A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES," *J. Biol. Chem.*, vol. 226, no. 1, pp. 497–509, May 1957, doi: 10.1016/S0021-9258(18)64849-5.
- [48] E. Molina Grima, E.-H. Belarbi, F. G. Acién Fernández, A. Robles Medina, and Y. Chisti, "Recovery of microalgal biomass and metabolites: process options and economics," *Biotechnol. Adv.*, vol. 20, no. 7, pp. 491–515, Jan. 2003, doi: 10.1016/S0734-9750(02)00050-2.
- [49] J.-Y. Lee, C. Yoo, S.-Y. Jun, C.-Y. Ahn, and H.-M. Oh, "Comparison of several methods for effective lipid extraction from microalgae," *Bioresour. Technol.*, vol. 101, no. 1, Supplement, pp. S75–S77, Jan. 2010, doi: 10.1016/j.biortech.2009.03.058.
- [50] A. P. Florentino de Souza Silva *et al.*, "Comparison of pretreatment methods for total lipids extraction from mixed microalgae," *Renew. Energy*, vol. 63, pp. 762–766, Mar. 2014, doi: 10.1016/j.renene.2013.10.038.
- [51] S. C. Cunha and J. O. Fernandes, "Extraction techniques with deep eutectic solvents," *TrAC Trends Anal. Chem.*, vol. 105, pp. 225–239, Aug. 2018, doi: 10.1016/j.trac.2018.05.001.
- [52] C. Lodeiro and J.-L. Capelo-Martínez, "Beyond Analytical Chemistry," in *Ultrasound in Chemistry*, John Wiley & Sons, Ltd, 2008, pp. 129–150. doi: 10.1002/9783527623501.ch6.
- [53] K. Kumar, S. Srivastav, and V. S. Sharanagat, "Ultrasound assisted extraction (UAE) of bioactive compounds from fruit and vegetable processing by-products: A review," *Ultrason. Sonochem.*, vol. 70, p. 105325, Jan. 2021, doi: 10.1016/j.ultsonch.2020.105325.

- [54] P. Das, P. K. Nayak, and R. krishnan Kesavan, "Ultrasound assisted extraction of food colorants: Principle, mechanism, extraction technique and applications: A review on recent progress," *Food Chem. Adv.*, vol. 1, p. 100144, Oct. 2022, doi: 10.1016/j.focha.2022.100144.
- [55] M. Roy *et al.*, "Integration of ultrasound-assisted extraction and deep eutectic solvent for enhanced recovery of bioactive compounds: Innovative green approach," *Food Chem. Adv.*, vol. 9, p. 101153, Dec. 2025, doi: 10.1016/j.focha.2025.101153.
- [56] Q. Ji, X. Yu, A. E.-G. A. Yagoub, L. Chen, O. A. Fakayode, and C. Zhou, "Synergism of sweeping frequency ultrasound and deep eutectic solvents pretreatment for fractionation of sugarcane bagasse and enhancing enzymatic hydrolysis," *Ultrason. Sonochem.*, vol. 73, p. 105470, May 2021, doi: 10.1016/j.ultsonch.2021.105470.
- [57] M. Češi *et al.*, "New insights into fatty acid-based deep eutectic solvent as a carrier for cannabidiol: Cytotoxic and genotoxic effects in human astrocytoma cells," *J. Mol. Liq.*, vol. 440, p. 128965, Dec. 2025, doi: 10.1016/j.molliq.2025.128965.
- [58] T. L. Riss *et al.*, "Cell Viability Assays," in *Assay Guidance Manual*, Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences, 2004. Accessed: Nov. 25, 2025. [Online]. Available: <http://www.ncbi.nlm.nih.gov/books/NBK144065/>
- [59] Promega Corporation, "CellTiter-Blue® Cell Viability Assay: Technical Bulletin TB317 (Revised 7/23)." Promega, 2023. [Online]. Available: https://at.promega.com/-/media/files/resources/protocols/technical-bulletins/101/celltiter-blue-cell-viability-assay-protocol.pdf?rev=3313091698e544ceaa6fff843658eb84&sc_lang=en
- [60] P. Skehan *et al.*, "New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening," *JNCI J. Natl. Cancer Inst.*, vol. 82, no. 13, pp. 1107–1112, Jul. 1990, doi: 10.1093/jnci/82.13.1107.
- [61] PubChem, "PubChem." Accessed: Jul. 12, 2025. [Online]. Available: <https://pubchem.ncbi.nlm.nih.gov/>
- [62] "Merck | Spain | Life Science Products & Service Solutions." Accessed: Jul. 12, 2025. [Online]. Available: <https://www.sigmaaldrich.com/ES/en>

- [63] M. S. Jovanović *et al.*, "Ultrasound-assisted natural deep eutectic solvents (NaDES) extraction of gentiopicoside, isovitexin, and isogentisin from *Gentiana asclepiadea* L. biowaste," *Sustain. Chem. Pharm.*, vol. 42, p. 101808, Dec. 2024, doi: 10.1016/j.scp.2024.101808.
- [64] W. Wojnowski, M. Tobiszewski, F. Pena-Pereira, and E. Psillakis, "AGREEprep – Analytical green-ness metric for sample preparation," *TrAC Trends Anal. Chem.*, vol. 149, p. 116553, Apr. 2022, doi: 10.1016/j.trac.2022.116553.
- [65] F. S. Costa *et al.*, "Microwave-assisted extraction based on emulsion breaking with natural deep eutectic solvent for vegetable oil sample preparation prior to elemental determination by ICP OES," *Talanta*, vol. 266, p. 125108, Jan. 2024, doi: 10.1016/j.talanta.2023.125108.
- [66] N. Castejón and F. J. Señoráns, "Simultaneous extraction and fractionation of omega-3 acylglycerols and glycolipids from wet microalgal biomass of *Nannochloropsis gaditana* using pressurized liquids," *Algal Res.*, vol. 37, pp. 74–82, Jan. 2019, doi: 10.1016/j.algal.2018.11.003.
- [67] Christie, W. W., "Preparation of Ester Derivatives of Fatty Acids for Chromatographic Analysis – AOCS," <https://www.aocs.org/>, 1993, Accessed: Jul. 13, 2025. [Online]. Available: <https://www.aocs.org/resource/preparation-of-ester-derivatives-of-fatty-acids-for-chromatographic-analysis/>
- [68] D. J. G. P. van Osch *et al.*, "A Search for Natural Hydrophobic Deep Eutectic Solvents Based on Natural Components," *ACS Sustain. Chem. Eng.*, vol. 7, no. 3, pp. 2933–2942, Feb. 2019, doi: 10.1021/acssuschemeng.8b03520.
- [69] K. Nowacki, M. Wysokowski, and M. Galiński, "Synthesis and characterization of betaine-based natural deep eutectic solvents for electrochemical application," *J. Mol. Liq.*, vol. 424, p. 127071, Apr. 2025, doi: 10.1016/j.molliq.2025.127071.
- [70] N. Ozel, A. Inam, and M. Elibol, "Exploring deep eutectic solvents for enhanced extraction of bio-active compounds from microalgae biomass," *J. Mol. Liq.*, vol. 407, p. 125237, Aug. 2024, doi: 10.1016/j.molliq.2024.125237.

- [71] C. Lo, J. Semerel, C. van den Berg, R. H. Wijffels, and M. H. M. Eppink, "Eutectic solvents with tuneable hydrophobicity: lipid dissolution and recovery," *RSC Adv.*, vol. 11, no. 14, pp. 8142–8149, Feb. 2021, doi: 10.1039/D1RA00306B.
- [72] Food Standards Agency and Food Standards Scotland, Eds., "Safety Assessment: Change of conditions of use for the novel food, *Schizochytrium* sp. oil rich in DHA and EPA," *FSA Res. Evid.*, Apr. 2024, doi: 10.46756/sci.fsa.pao657.
- [73] "Sax's Dangerous Properties of Industrial Materials, 11th Edition | Wiley." Accessed: Dec. 04, 2025. [Online]. Available: <https://www.wiley.com/en-us/Sax's+Dangerous+Properties+of+Industrial+Materials%2C+11th+Edition-p-9780471701347>
- [74] WHO/FAO Joint Expert Committee on Food Additives (JECFA), "Evaluation of certain food additives: Eighty-second report of the Joint FAO/WHO Expert Committee on Food Additives.," Geneva, WHO Technical Report Series, No. 1000., 2017.
- [75] A. Narayanan *et al.*, "Anticarcinogenic Properties of Medium Chain Fatty Acids on Human Colorectal, Skin and Breast Cancer Cells in Vitro," *Int. J. Mol. Sci.*, vol. 16, no. 3, pp. 5014–5027, Mar. 2015, doi: 10.3390/ijms16035014.
- [76] W. Thammasut *et al.*, "Antimicrobial and antitumoral activities of saturated fatty acid solutions," *Mater. Today Proc.*, Apr. 2023, doi: 10.1016/j.matpr.2023.03.769.
- [77] Joint FAO/WHO Expert Committee on Food Additives (JECFA) and World Health Organization (WHO), "Safety Evaluation of Certain Food Additives," Geneva, 1999.
- [78] World Health Organization and Food and Agriculture Organization of the United Nations, "Evaluation of certain food additives: eighty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives.," Geneva, WHO technical report series; no. 1014.
- [79] A. Dolghi *et al.*, "Chemical and Antimicrobial Characterization of *Mentha piperita* L. and *Rosmarinus officinalis* L. Essential Oils and In Vitro Potential Cytotoxic Effect in Human Colorectal Carcinoma Cells," *Molecules*, vol. 27, no. 18, p. 6106, Sep. 2022, doi: 10.3390/molecules27186106.

- [80] Berufsgenossenschaft der chemischen Industrie, "Toxikologische Bewertung," Heidelberg, 259, 2000.
- [81] Joint FAO/WHO Expert Committee on Food Additives (JECFA), "Evaluation of Certain Food Additives and Contaminants," Geneva, Technical Report WHO Technical Report Series 901, 2001.
- [82] Q. Zeng, Y. Che, Y. Zhang, M. Chen, Q. Guo, and W. Zhang, "Thymol Isolated from *Thymus vulgaris* L. Inhibits Colorectal Cancer Cell Growth and Metastasis by Suppressing the Wnt/ β -Catenin Pathway," *Drug Des. Devel. Ther.*, vol. 14, pp. 2535–2547, Jul. 2020, doi: 10.2147/DDDT.S254218.
- [83] "Carvacrol," *Food Cosmet. Toxicol.*, vol. 17, pp. 743–745, Dec. 1979, doi: 10.1016/S0015-6264(79)80018-8.
- [84] A. PAKDEMİRLİ *et al.*, "Carvacrol alters soluble factors in HCT-116 and HT-29 cell lines," *Turk. J. Med. Sci.*, vol. 50, no. 1, pp. 271–276, Feb. 2020, doi: 10.3906/sag-1907-173.
- [85] Merck Life Science / Sigma-Aldrich, "Safety Data Sheet: Poly(propylene glycol) bis(2-aminopropyl ether)." 2024. Accessed: Dec. 04, 2024 AD. [Online]. Available: <https://www.sigmaaldrich.com/>

8 Appendices

Table A 1: Factor levels and Box–Behnken experimental design matrix for the ultrasound-assisted extraction performed using an ultrasonic bath.

The table lists the coded and actual levels of the three independent variables (time, temperature, and molar ratio), along with the 15 randomized experimental runs including three replicated center points.

	Time (min)	Amplitude (%)	Pulse (%)
Lower (−1)	2	40	40
Middle (0)	6	65	70
Upper (+1)	10	90	100
1	2	90	70
2	2	65	40
3	2	65	100
4	2	40	70
5	6	90	40
6	6	40	40
7	6	40	100
8	6	90	100
9	6	65	70
10	6	65	70
11	6	65	70
12	10	90	70
13	10	65	40
14	10	40	70
15	10	65	100

Table A 2: Factor levels and Box–Behnken experimental design matrix for the ultrasound-assisted extraction performed using an ultrasonic probe.

The table includes the coded and actual levels for time, amplitude, and pulse, as well as the 15 randomized experimental runs with three replicated center points.

	Time (min)	Temperature (°C)	molar ratio

Lower (-1)	10	30	1:2
Middle (0)	20	45	1:1
Upper (+1)	30	60	2:1
1	10	45	1:2
2	20	60	1:2
3	20	30	1:2
4	30	45	1:2
5	30	60	1:1
6	20	45	1:1
7	20	45	1:1
8	20	45	1:1
9	10	30	1:1
10	10	60	1:1
11	30	30	1:1
12	10	45	2:1
13	30	45	2:1
14	20	30	2:1
15	20	60	2:1

Table A 3: Coded regression coefficients of the Box–Behnken response surface model for the Me:Th ultrasonic bath extraction.

Coded Coefficients

	Value	Standard Error	95% LCL	95% UCL	t-Value	Prob > t	VIF
(Intercept)	53.95589	1.11968	51.68283	56.22896	48.18874	1.36136E-33	--
Time	2.05422	0.68566	0.66225	3.44618	2.99597	0.005	1
Temperature	1.24763	0.68566	-0.14433	2.6396	1.81961	0.07738	1
Molar Ratio	0.98167	0.68566	-0.41029	2.37364	1.43172	0.1611	1
Time*Time	-0.27563	1.00926	-2.32455	1.77328	-0.2731	0.78638	1.01111
Temperature*Temperature	-0.94856	1.00926	-2.99748	1.10035	-0.93985	0.35373	1.01111
Molar Ratio*Molar Ratio	-2.98279	1.00926	-5.0317	-0.93387	-2.95541	0.00556	1.01111
Time*Temperature	-3.25464	0.96967	-5.22317	-1.28611	-3.35644	0.00191	1
Time*Molar Ratio	-0.40851	0.96967	-2.37704	1.56003	-0.42128	0.67612	1
Temperature*Molar Ratio	-0.14685	0.96967	-2.11539	1.82168	-0.15145	0.88049	1

Table A 4: Model statistics for the response-surface model describing Me:Th extraction using the ultrasonic bath.

Statistics

	mg FA/g extract
Degress of Freedom	35
Root Mean Square of Error	3.35904
R-Square	0.50034
Adj. R-Square	0.37186
PRESS	690.13429
Residual Sums of Squares	394.90919
Predicted. R-Square	0.13235

Table A 5: ANOVA results for the Box–Behnken response surface model of the Me:Th ultrasonic bath extraction.

ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Time	1	101.27523	101.27523	8.97582	0.005
Temperature	1	37.35799	37.35799	3.31096	0.07738
Molar Ratio	1	23.12826	23.12826	2.04981	0.1611
Time*Time	1	0.84156	0.84156	0.07459	0.78638
Temperature*Temperature	1	9.96666	9.96666	0.88332	0.35373
Molar Ratio*Molar Ratio	1	98.55168	98.55168	8.73444	0.00556
Time*Temperature	1	127.11217	127.11217	11.26569	0.00191
Time*Molar Ratio	1	2.00253	2.00253	0.17748	0.67612
Temperature*Molar Ratio	1	0.25879	0.25879	0.02294	0.88049
Error	35	394.90919	11.28312		
Lack of fit	3	67.06421	22.35474	2.18198	0.10939
Pure Error	32	327.84497	10.24516		
Total	44	790.35841			

Table A 6: Model-derived optimum extraction parameters for Me:Th in the ultrasonic bath.

Solution

	Value
Time	30
Temperature	30
Molar Ratio	1.04:1
Composite desirability: 0.83886	

Table A 7: Model prediction of fatty acid yield and desirability at the optimized extraction settings for the ultrasonic bath.

Prediction

	Fit	SE of Fit	95%LCL	95%UCL	95%LPL	95%UPL	Desirability
mg FA/g extract	56.83638	1.68569	53.41425	60.2585	49.20666	64.46609	0.83886

Table A 8: Coded regression coefficients of the Box–Behnken response surface model for the Me:Th ultrasonic probe extraction.

Coded Coefficients

	Value	Standard Error	95% LCL	95% UCL	t-Value	Prob > t	VIF
(Intercept)	45.00148	1.99963	40.94202	49.06094	22.50494	2.10911E-22	--
Time	9.12599	1.22452	6.64009	11.61189	7.45273	1.00464E-8	1
Amplitude	13.12538	1.22452	10.63948	15.61128	10.71883	1.33672E-12	1
Pulse	7.46268	1.22452	4.97678	9.94858	6.09439	5.80021E-7	1
Time*Time	-4.09204	1.80244	-7.75118	-0.43289	-2.27028	0.02946	1.01111
Amplitude*Amplitude	-6.36193	1.80244	-10.02108	-2.70278	-3.52962	0.00119	1.01111
Pulse*Pulse	-1.17394	1.80244	-4.83309	2.4852	-0.65131	0.5191	1.01111
Time*Amplitude	-0.19612	1.73173	-3.71172	3.31947	-0.11325	0.91048	1
Time*Pulse	0.35141	1.73173	-3.16418	3.86701	0.20293	0.84037	1
Amplitude*Pulse	-2.07185	1.73173	-5.58745	1.44374	-1.19641	0.23958	1

Table A 9: Model statistics for the response-surface model describing Me:Th extraction using the ultrasonic probe.

Statistics

	mg FA/g extract
Degress of Freedom	35
Root Mean Square of Error	5.99888
R-Square	0.86569
Adj. R-Square	0.83116
PRESS	2067.75505
Residual Sums of Squares	1259.5303
Predicted. R-Square	0.78077

Table A 10: ANOVA results for the Box–Behnken response surface model of the Me:Th ultrasonic probe extraction.

ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Time	1	1998.80878	1998.80878	55.54317	1.00464E-8
Amplitude	1	4134.61663	4134.61663	114.89329	1.33672E-12
Pulse	1	1336.59869	1336.59869	37.14159	5.80021E-7
Time*Time	1	185.4805	185.4805	5.15416	0.02946
Amplitude*Amplitude	1	448.32888	448.32888	12.45822	0.00119
Pulse*Pulse	1	15.26562	15.26562	0.4242	0.5191
Time*Amplitude	1	0.46156	0.46156	0.01283	0.91048
Time*Pulse	1	1.4819	1.4819	0.04118	0.84037
Amplitude*Pulse	1	51.51096	51.51096	1.43139	0.23958

Error	35	1259.5303	35.98658		
Lack of fit	3	277.13986	92.37995	3.00915	0.04454
Pure Error	32	982.39044	30.6997		
Total	44	9378.00676			

Table A 11: Model-derived optimum extraction parameters for Me:Th in the ultrasonic probe.

Solution

	Value
Time	10
Amplitude	86
Pulse	100
Composite desirability: 0.85612	

Table A 12: Model prediction of fatty acid yield and desirability at the optimized extraction settings in the ultrasonic probe.

Prediction

	Fit	SE of Fit	95%LCL	95%UCL	95%LPL	95%UPL	Desirability
mg FA/g extract	61.30796	3.74092	53.71348	68.90244	46.95564	75.66028	0.85612

Table A 13: Results of Dunnett's post hoc test for the CTB assay of NaDES (solvents) (HCEC-1CT). Mean differences, test statistics (q values) and corresponding p values are shown for each treatment compared to the DMSO control (0.25 % v/v). Significant differences ($p < 0.05$) are indicated by "yes" in the Sig. column, non-significant differences by "-". "Viability < 80 %" marks biologically relevant cytotoxicity and is indicated in the same manner.

Dunnett Test					
Treatment	Mean Difference	q Value	p Value	Sig. ($p < 0.05$)	Viability < 80 %
C8:C12 1 $\mu\text{g mL}^{-1}$	-0.01174	0.43227	1	—	
C8:C12 10 $\mu\text{g mL}^{-1}$	-0.02321	0.85481	0.99707	—	
C8:C12 100 $\mu\text{g mL}^{-1}$	-0.12597	4.63901	2.51542E-4	yes	—
C8:Th 1 $\mu\text{g mL}^{-1}$	-0.01769	0.65149	0.9999	—	
C8:Th 10 $\mu\text{g mL}^{-1}$	0.01745	0.64271	0.99992	—	
C8:Th 100 $\mu\text{g mL}^{-1}$	-0.00913	0.33618	1	—	
C8:Me 1 $\mu\text{g mL}^{-1}$	0.01757	0.64699	0.99991	—	
C8:Me 10 $\mu\text{g mL}^{-1}$	-0.00619	0.22784	1	—	
C8:Me 100 $\mu\text{g mL}^{-1}$	0.00921	0.33925	1	—	
C8:Ca 1 $\mu\text{g mL}^{-1}$	0.03059	1.12647	0.95933	—	
C8:Ca 10 $\mu\text{g mL}^{-1}$	0.08493	3.12765	0.03253	yes	—
C8:Ca 100 $\mu\text{g mL}^{-1}$	0.03438	1.26602	0.90619	—	
Me:Th 1 $\mu\text{g mL}^{-1}$	0.03817	1.40548	0.82722	—	

Me:Th 10 $\mu\text{g mL}^{-1}$	0.02312	0.85136	0.9972	–	
Me:Th 100 $\mu\text{g mL}^{-1}$	-0.03849	1.41749	0.8194	–	
Comm. Oil 1 $\mu\text{g mL}^{-1}$	0.04003	1.47399	0.78088	–	
Comm. Oil 10 $\mu\text{g mL}^{-1}$	0.09357	3.44601	0.01323	yes	–
Comm. Oil 100 $\mu\text{g mL}^{-1}$	0.13871	5.10813	<0.0001	yes	–
0.1 % Triton X	-0.98692	36.34479	<0.0001	yes	yes