Bachelor's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Emilie Grøntveit Tverå

Assessing the feasibility of applying natural deep eutectic solvents for the solubilisation of collagen

Bachelor's thesis in Food Science, Technology and Sustainability
Supervisor: Ashkan Madadlou
May 2023

NTNU
Norwegian University of
Science and Technology

Emilie Grøntveit Tverå

Assessing the feasibility of applying natural deep eutectic solvents for the solubilisation of collagen

Bachelor's thesis in Food Science, Technology and Sustainability
Supervisor: Ashkan Madadlou
May 2023

Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

NTNU
Norwegian University of
Science and Technology

NTNU - Norwegian University of Science and Technology
Department of Biotechnology and Food Science

BACHELOR'S THESIS 2023

15 ECTS

Assessing the feasibility of applying natural deep eutectic solvents for the solubilisation of collagen

by

Emilie Grøntveit Tverå

Abstract

Collagen is an important biological material desired in biomedicine, pharmaceuticals, and in the food industry for its versatile properties. The conventional extraction method applied in the industry today is a timely process, which includes multiple rounds of treatment in acidic or alkaline environments, and excessive purification steps. In this work, natural deep eutectic solvents (NADESs) are explored for the solubilisation of collagen as a greener alternative to the conventional method. Two NADES formulations were chosen, citric acid : xylitol : water (CA-X-W) and choline chloride : oxalic acid dihydrate (ChCl-OAD), and they were tested on representative sample materials to explore their potential properties for extraction of collagen. Where gelatin represents the collagen peptide sequence, and cellulose the macrofibre structure. Different assays were performed to assess for their solubilisation efficacy, solvent attributes, and the characteristics of the solubilised samples. CA-X-W solubilised 99.5% of the gelatin sample and caused a high degree of dissolution of the collagen molecules. It was also observed to be non-destructive to the collagen molecules and is thus the most promising solvent for application in an industrial extraction. ChCl-OAD solubilised 66.1% of the gelatin sample and stood out for having an excessive proteolytic effect on the collagen molecules, creating small peptides. The extraction product would likely not have the sought gelation properties of collagen but could potentially be utilised in fields other than the food industry. This project has overall provided significant information about these solvents, and this knowledge will provide a sound basis for further research on the topic of applying NADESs in extraction processes for collagen.

Sammendrag

Kollagen er et viktig biologisk materiale av stor etterspørsel innen biomedisin, farmasøytisk industri og i næringsmiddelindustrien for sine allsidige egenskaper. Den konvensjonelle ekstraksjonsmetoden som benyttes i industrien i dag er en tidskrevende prosess, som inkluderer flere runder med behandling i sure eller alkaliske miljøer, og lengre rensetrinn. I dette arbeidet utforskes det om løsemidlene «natural deep eutectic solvents» (NADES) kan benyttes som et grønnere alternativ til den konvensjonelle metoden for oppløsningen av kollagen. To typer NADES ble valgt ut, sitronsyre : xylitol : vann (CA-X-W) og kolinklorid : oksalsyre dihydrat (ChCl-OAD), og disse ble testet på representative prøvematerialer for å utforske deres potensielle egenskaper for ekstraksjon av kollagen. Gelatin ble brukt for å ha primærstrukturen til kollagen, og cellulose representerer makrofiberstrukturen. Ulike analyser ble utført for å vurdere grad av oppløst prøvemateriale, løsemidlenes egenskaper og egenskapene til de oppløste prøvene. CA-X-W løste opp 99.5 % av gelatinet og forårsaket en høy grad av oppløsning av kollagenmolekylene. Løsemiddelet ble også observert å ikke ha destruktivt effekt på kollagenmolekylene og er dermed det mest lovende løsningsmidlet for bruk i en industriell ekstraksjon. ChCl-OAD løste opp 66,1 % av gelatinprøven og skilte seg ut ved å ha en overdreven proteolytisk effekt på kollagenmolekylene, noe som skapte små peptider. Ekstraksjonsproduktet vil sannsynligvis ikke ha de ettersøkte geleringsegenskapene til kollagen, men kan potensielt ha bruksområder innen andre felt enn næringsmiddelindustrien. Dette prosjektet har totalt sett samlet betydelig informasjon om disse løsningsmidlene, og denne kunnskapen vil gi et godt grunnlag for videre forskning på bruk av NADES i ekstraksjonsprosesser for kollagen.

Preface

This work was conducted as the concluding remarks of my bachelor's degree in Food Science, Technology and Sustainability at the Department of Biotechnology and Food Science at the Norwegian University of Science and Technology (NTNU) in Trondheim. The thesis was conducted in the Spring of 2023 under the supervision of Ashkan Madadlou from the Department of Biotechnology and Food Science

I would like to extend my sincere thanks to my supervisor, Ashkan Madadlou, for the insightful and encouraging discussions throughout the thesis, and for all the help and guidance. It has truly boosted my confidence in my own abilities.

My heartfelt appreciation goes to my lovely partner, Saeid Shamsaliei. Throughout the course of this thesis, you have been an unwavering source of strength and compassion.

To my family, I am grateful for your support and encouragement throughout my academic journey.

Lastly, I would like to thank me. I did it, good job.

22.05.2023, Trondheim

1 TABLE OF CONTENTS

2 INTRODUCTION

2.1 COLLAGEN – PROPERTIES, STRUCTURE AND USES

Collagen is a fibrous protein and the primary structural element in connective tissues (Shoulders & Raines, 2009). It is the most abundant protein in animals and dominates the constitute of skin and bones. The protein structure is strong and stable, giving the tissues its characteristic properties. As of now there is known to be at least 28 different types of collagens, each with their own distinct monomer peptide chain, and with type I being the most abundant (Matinong et al., 2022). Although each collagen type has their own unique traits, there is a certain pattern that all collagens follow strongly in the peptide sequence. It is found that every third residue in collagen is glycine, giving a Xaa-Yaa-Gly sequence, where the X and Y residues vary. Any residue can occupy the X and Y positions, but usually it is proline and 4-hydroxyrpoline. As shown in Figure 1, these collagen subunits are arranged together as three left-handed polypeptide α-chains, forming a triple right-handed super helix structure, which is known as tropocollagen (Shoulders & Raines, 2009). Each of these single chains are usually around 300 kDa, with each chain containing about 1020 amino acid residues (Gelse, 2003). The peptide chains in tropocollagen are held together by hydrogen-bonding between the N-H in glycine the and O=C in Xaa. When many of these lay parallel to each other, with a slight displacement, it gives the formation of microfibrils, and then on the last hierarchical level, those will form a larger network which is the collagen fibre (Shoulders & Raines, 2009).

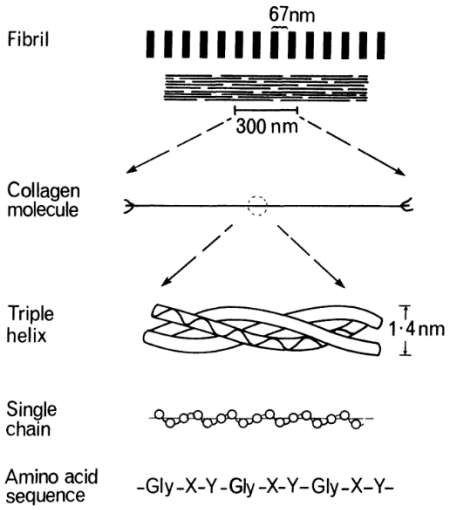


Figure 1: The figure shows an overview of the hierarchal collagen structure, starting with the Gly-X-Y amino acid sequence forming a helical chain. Three of these come together to form a triple helix, which is the collagen molecule. The final collagen fibril is constituted of multiple collagen molecules held together by hydrogen bonding on all levels. (From: (Djabourov et al., 1988)*)*

2.1.1 The conventional extraction process

Collagen is practically insoluble in water because its isoelectric point is very close to neutral pH, which allows excessive hydrogen bonding (Telis et al., 2006; Yang et al., 2020). At acidic conditions, the protein becomes positively charged, causing repulsion between the collagen molecules, thus

disrupting the structure. The conventional extraction process of collagen follows this principle closely, involving pre-treatments steps, extraction steps, and lastly, purification of the collagen. The purpose of pre-treatment is to break the intermolecular crosslinks between the collagen molecules in the collagen fibres by hydrolysis, which leaves the tropocollagen intact (Yang et al., 2020). One way to do this is by acid. In this case, the biomass samples would be immersed in dilute acid, resulting in the fibres swelling. This method is quite gentle and is most suitable for fragile skins such as fish and porcine (Schmidt et al., 2016). The other pre-treatment is alkaline, using bases, which can take days to weeks to complete (Yang et al., 2020). Here, the treatment works by hydrolysing unwanted, non-collagenous protein, lipids, and other materials, and is more suited for thick and hard materials (Benjakul et al., 2009; Pal & Suresh, 2016). After pre-treatment of the sample, the collagen itself can be extracted through multiple different ways. The first method is by acid hydrolysis where samples soak for 24-72 hours while continuously stirring at low temperatures to minimize degradation. Throughout the extraction step hydrogen bonds will be further cleaved, through creating the net positive charge that promotes repulsion. Alternative extraction methods include creating an alkali environment, using salts, and utilizing enzymatic hydrolysis. After extraction, the collagen is then precipitated using salts, which leave a product in need of purification. The purification step involves removing non-collagen proteins by salting the target protein out, followed by 4-10 days of dialysis to remove all the added salts (Yang et al., 2020). Lastly the product is dried. What is left is essentially a product of fully or partially hydrolysed collagen molecules, with the individual peptide chains still intact. Commercially, this product is often called gelatin.

From this overview, it is obvious that the extraction process is quite inconvenient having multiple treatments and long extraction times (Batista et al., 2022). Purification alone takes up nearly half of the total time, making the whole process around 2 weeks. Adding to down-sides is the fact that a lot of chemical waste is created, which are quite corrosive to the equipment, and the whole process ends up being very energy consuming (Matinong et al., 2022).

2.1.2 Demand and applications

Even if the process is inconvenient, collagen is a material of high interest in the industry. Most of the demand today is found within food production, pharmaceuticals, and biomedicine, and is estimated to steadily increase globally over the next few years (Gnanamani et al., 2020). The demand is owed to collagen holding many desirable properties. Firstly, collagen has a very high capacity for water absorption, making it capable of texturing, thickening, and gelling. Secondly, it can be utilized for its versatile surface behaviour, for emulsions, foaming, stabilisation, adhesion, and film forming, just to mention some. And lastly, its biocompatibility and degradability make collagen very attractive as a

potential biomedical application as it could result in favourable interactions in the body and degrade when its use is done. (Coppola et al., 2020).

Most collagen today is extracted from porcine and bovine sources, which leads to a few issues. Firstly, bovine sources pose a risk of transmitting zoonotic disease, while secondly, porcine sources may present conflict among some religious communities (Bisht et al., 2021). It is therefore of interest to look at other sources of collagen to meet the large demand as well as adjusting to consumer preferences. Some has looked at marine sources such as fish biomass as a potential alternative, and it is showing promising results as a substitute (Batista et al., 2022; Bisht et al., 2021). Research is already exploring how marine collagen can be used for films in food packaging and as bioactive peptides (Coppola et al., 2020). Expanding on this, Wang et al (2017) found that collagen derived from fish skin could favourably integrate to tissue and blood vessels, showing properties as a scaffolding material in biomedical applications. Besides the beneficial applications of fish collagen, using fish biomass would also be of environmental interest. Skin, bones, and scales as waste is shown to make up to 25% of fish production, which are also the most collagen abundant constituents of the fish (Coppola et al., 2020). Reducing this waste would therefore be of massive importance environmentally, as well as resulting in economic gain for the industry.

As mentioned, though there is an interest in increasing the collagen production, the extraction method used today is very inconvenient. As an alternative to this, many are now exploring a new type of solvent called “deep eutectic solvent”, also referred to as “DES”.

2.2 DEEP EUTECTIC SOLVENTS

This chapter explores what deep eutectic solvents are, including their use and properties. A sub-category called “natural” deep eutectic solvents will also be defined, as well as how they can used for collagen extraction.

2.2.1 The structure of DESs

Deep eutectic solvents, hereby referred to as DESs, are considered as cheap and safe solvents, consisting only of two to three components. The components are mixed, where one component works as the hydrogen bond donor (HBD), whilst the other as the hydrogen bond acceptor (HBA). If the DES system has a third component, it is in most cases water, but other substances may also be used (Q. Zhang et al., 2012). During early research on DESs, only halide salts such as choline chloride were used as HBA, while the HBD would mostly be alcohol, amides, and carboxyl acids (El Achkar et al., 2021; Q.

Zhang et al., 2012). Today though, DES formulations with a larger variety of different compounds have been discovered, making them less restrictive. Even with all these new components, choline chloride is still very frequently used as the HBA, seeing as it is very cheap, biodegradable, and non-toxic, making it suitable for many applications (El Achkar et al., 2021).

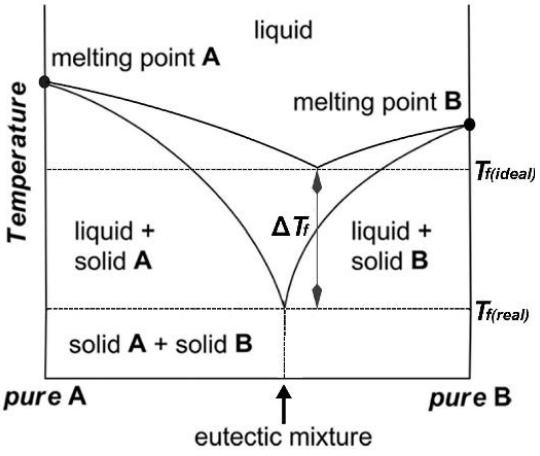


Figure 2: The phase diagram shows the melting point of the mixture being a function of the ratio of two components where the far left and the far right of the x-axis each represents the composition being of one pure component. The eutectic point is shown for when the melting point is at its lowest. (From: (Kalhor & Ghandi, 2019))

The components of DESs interact through hydrogen bonding, forming a eutectic mixture. Eutectic mixtures are characterized by having melting points lower than those of the individual components they consist of. The decrease in melting point is caused by the charges in the compounds being delocalized by the hydrogen bonding (Paiva et al., 2014). The stronger the hydrogen bond interactions between HBD and HBA, the lower the melting point. The eutectic point is the molar ratio of HBA and HBD at which the melting point is the lowest, meaning there is a perfect ratio where the hydrogen bonds have the strongest interaction with each other. This phenomenon is graphically shown in Figure 2. Most of these mixtures are liquid between room temperature and 70°C, and they all have a freezing point lower than 150°C (Q. Zhang et al., 2012).

There are many ways to define DESs, and the one used today is considerably less strict than it used to be some years ago, owing to the extensive research done on these liquids. For example, a DES does not have to be made at the exact eutectic point, if the DES stays liquid at operating temperature, it will still be regarded as a DES. This allows more customizability of the DES properties as molar ratio can decide many physiochemical factors. Furthermore, the DES system itself is often described as Cat+ X- zY, where Cat+ is an ammonium, phosphonium, or sulfonium cation, X- is a Lewis base (halide anion), Y is a Lewis / Brønsted acid, and z is the number of Y interacting with X-. To give an example, choline would be Cat+, and chloride is X-, together being the HBA. While oxalic acid can be Y, the HBD, and z is 1, meaning the molar ratio is 1:1. With broad definitions such as these it is now easier to find new DESs, and to modify them to the purpose of interest. (El Achkar et al., 2021)

2.2.1.1 Synthetisation

There are a plenty of methods to make DES. However, a few main ones are more common. First, it is important to note that making a DES requires no other chemicals than the components it consists of,

which results in a 100% output with no need for purification (Q. Zhang et al., 2012). The first method is the evaporation method. Here, the components are dissolved in water and then combined. The solution is then evaporated at 50°C in a rotary evaporator until the water is completely removed and the finished solvent is then placed in a desiccator. The second method of making DESs is the heating method. Here the components are mixed and stirred together while being heated until a homogenous and clear liquid is formed. Water or oil baths are usually used at heating, and the temperature is usually between 50°C and 80°C (Dai et al., 2013). Increasing the temperature would allow the components to melt quicker, but it is important to note that too high temperatures may lead to degradation of the DES (Rodriguez et al., 2019). Both methods are quite easy, which allows them to be applied in high production volumes of DESs.

A few other methods have also been explored, such as ultrasound-assisted and freeze-drying assisted synthesis, where the latter involves dissolving the components together in water before freeze-drying them until a transparent liquid is obtained (Santana et al., 2019). Microwave-assisted synthesis has also been explored as a promising green alternative to those already in use. With the synthetisation time down to less than 1 minute, it is by far the fastest one. There is, of course, a risk of overheating, so this method should optimally be done in several cycles of few seconds at a time until a homogeneous and clear liquid is formed (Gomez et al., 2018).

2.2.1.2 The favourability and appeal of DESs

When comparing DESs to the other potential solvents for the extraction of collagen, it becomes obvious that DESs has many advantages. A different solvent known as ionic liquids (ILs) has been explored for a longer period than DESs. In fact, they are both quite similar, as ILs are made by mixing two salts, which also creates a eutectic mixture. They also have in common that they have a broad range of customizability of their properties. What drives research towards using DESs instead of ILs though has to do with the fact that ILs are quite toxic and have poor degradability (Q. Zhang et al., 2012). Additionally, the synthesis requires large amounts of salts and solvents, and afterwards the solvent must be purified multiple times, which is bad for economic and environmental sustainability (Rashid et al., 2023). Comparably, the DESs have a much easier synthetisation process, which is supported by its convenient atom economy, high yield of production, low volatility, low melting points, non-flammability, polarity, and chemical and thermal stability (Smith et al., 2014; Q. Zhang et al., 2012)

2.2.1.3 Applications

There are many potential uses for DESs that are being explored in recent works, owing to their physiochemical properties. For example, DES can be applied as a solvent in catalytic processes to allow

good contact between reactants and catalysts, as electrolytes and solvents in electrochemical reactions, in the preparation of new materials, and for the dissolution of valuable substrates such as inorganic salts and metal oxides (Q. Zhang et al., 2012). Furthermore, Kalhor & Ghandi (2019) has explored how DES can be used as pre-treatment solvents and as extraction solvents, which here is the sought application on collagen. In terms of pre-treatments, DES were successfully used to break down and open the structure of wheat straw, increasing the access hydrolysing enzymes had to the inner structure (Zhao et al., 2018). Whilst for extractions, a DES of choline chloride : malic acid composition was used to extract chitin from shrimp shells, resulting in a 19% yield, which is considerably higher than conventional chitin extraction methods (Huang et al., 2018). An important factor for extraction processes with DESs is shown to be temperature. Increasing the temperature would increase the molecular mobility, meaning that the extracted molecules diffuse into the solvent quicker (Skarpalezos & Detsi, 2019). Something that adds to the extraction properties of DESs, is its recyclability. The same DES can be used as a solvent for multiple rounds of extraction, only needed the dissolved material to be separated out in between, and in some cases, purification. There was even reported no loss of performance after three cycles of reusing the same solvent for pre-treatment of rice-straw (Kumar et al., 2016), while others report that the solubility and thermal stability may decrease slightly (Kalhor & Ghandi, 2019). Implementing a recycling procedure for the DESs after extraction could contribute to reducing the waste. To sum it up, there are many possible applications of DESs, and studies show that they often give better results than conventional methods, which leads to the interest to see if these solvents also can be applied for the solubilisation of collagen in an extraction process.

2.2.1.4 Properties as solvents

There is still a vast lack of understanding regarding how DESs are formed, and their properties in different applications such as solvents (El Achkar et al., 2021). According to Abbott et al (2003), the solvent properties of DESs are strongly influenced by hydrogen bonding. Meaning that high solubility properties are shown for compounds capable of accepting or donating electron or protons to create hydrogen bonds with the target substance. It is also stated that polarity plays an important role. DESs are generally quite hydrophilic due to high electronegativity and hydrogen bonding via dipole-dipole interactions, making them comparable to polar solvents (Liu et al., 2018). The degree of polarity the DES has depends on its constituents, and some DESs are observed to both have polar and non-polar properties (Benvenutti et al., 2019). Craveiro et al (2016) found that DESs consisting of choline chloride and an organic acid were more polar and had better solvent properties than DESs consisting of choline chloride and pure sugars. More polarity does not necessarily mean increased solubility though, because it is more about the likeness in polarity with the target extraction material. Research done on

the extraction of flavonoids showed that it is the DES with the most similar polarity to the target substance that resulted in the highest extraction efficacy (Z. Meng et al., 2018; Yao et al., 2015). Therefore, when wanting to formulate a possible DES for a wanted material it would be beneficial to try and match the polarity of the material. In general, there are some DES components that have a big impact on deciding the polarity. Organic acid – based DESs are found to be the most polar, followed by the amino acid based, and then pure sugar based, which have a polarity close to water (Dai et al., 2013). But of course, there are other factors that may reduce the solubilising ability even if the polarity is optimal, such as suboptimal molar ratio in the DES. Another factor which may influence the solubilisation efficacy of DES is the addition of water. This is mainly due to the reduction in viscosity, which increases the mass transfer of solubilisation. Water might also change the polarity in some cases. Depending on the specific DES, the polarity may both increase and decrease if diluted with water (Dai et al., 2013), which would mean that water addition into a DES might be beneficial to the technical properties of the DES but could potentially have a negative impact on the solubilisation properties.

2.2.1.5 Other properties of DESs

The physiochemical properties of DESs, including viscosity, melting point, density, ion conductivity and surface tension, can all be modified and adjusted to fit specific applications (El Achkar et al., 2021). The addition of water is applied in many instances to modify the properties. Melting point and viscosity linearly decrease as a function of water content, while the conductivity increases notably (Du et al., 2016; X. Meng et al., 2016). As of now, there are two ways water has been observed to interact with the DES system, as a structural component, or as a diluent. This property is quite important to study further as water can disturb the intra- and intermolecular bonds in the eutectic system, and therefore disrupt its integrity. Some researchers see the addition of water to a DES system as a gradient going from a “water in DES” (structural) system, to a “DES in water” (aqueous solution) system (Hammond et al., 2017). It is not clear when that changeover exactly happens, but it depends on what the HBD and HBA are, and at which molar ratio they are mixed (Gabriele et al., 2019). In a “water in DES” system, water act as a structural component and will interact as an HBD. This is quite favourable as the water becomes part of the DES network and will strengthen the hydrogen bonds by placing itself between the main HBA and HBD components (Hammond et al., 2017). On the other hand, there is the “DES in water” system which weakens the interactions in the DES supramolecular structure as water will interact with the constituent compounds rather than joining the system. This dilution therefore results in a loss of the existing hydrogen bonds, also meaning the eutectic structure is lost. The system instead becomes an aqueous solution of dissolved components in water (Dai et al., 2013).

It is not just the amount of water that decides if it acts as a structural component of the DES or as a diluent. Water that is added during the making of DES usually ends up as structural water, while water added later will usually act as diluent water. This is something that might explain why results often differ from one paper to the next regarding physiochemical properties, since the time of water addition could play a big role. Especially seeing that these chemicals usually have a natural affinity for water, which could mean that there already is some water present from the beginning unaccounted for if the chemicals are not dried beforehand. (Lorenzetti et al., 2022)

Viscosity has been mentioned before, and that owes to its importance in a practical sense. When a DES has high viscosity, it is most likely due to the excessive hydrogen bonding between the components, and to some degree van der Waals and electrostatic interactions. This results in lower mobility of free species, and therefore, high viscosity (Q. Zhang et al., 2012). High viscosity is seen as an inconvenient trait in applications as it causes low mass transfer, which affects the extraction efficiency (Rente et al., 2021). There are a few ways to adjust the viscosity, which is through the choice of components, molar ratio, temperature, and water content (Q. Zhang et al., 2012).

Ionic conductivity is a different property that has been explored. Generally, among DESs, the conductivity is low due to the high viscosity, but that also means that a decrease in the viscosity would increase the conductivity (Q. Zhang et al., 2012). There is also some DES that are naturally highly conductive, which confirms that the ionic species in the DESs are dissociated and can move independently (Abbott et al., 2003).

When wanting to define the pH of a DES, it gets difficult. Using a normal pH-meter would probably give an inaccurate result as they are meant to be used on aqueous solutions. A proposed alternative is to calculate the pH, which can be done by the Hammett acidity function (Q. Zhang et al., 2012). This is a thermodynamic quantitative measure which is used to find the acid strength of non-aqueous solutions and very concentrated Brønsted acids (S. Zhang et al., 2016).

Lastly, the stability of a DES will vary depending on the situation. One factor that affects this can quite simply just be the molar ratio of the DES components. E.g., while a 1:1 ratio becomes a stable liquid, a 1:2 ratio might be liquid at first but will gradually form crystalline precipitate. The composition itself is also a factor here, as components capable of forming many hydrogen bonds will be able to form stable DES with a higher number of other components than those that can only form a few bonds. Citric acid, for instance, can form stable liquids with lots of different components compared to for example malic acid, which cannot form as many hydrogen bonds (Dai et al., 2013).

2.2.2 Natural deep eutectic solvents

Natural deep eutectic solvents, forthwith referred to as “NADESs”, was a term first introduced in 2011 by Choi et al (2011) while exploring eutectic mixtures in nature. These are a subcategory of DES where the components consist of primary metabolites from natural resources. There are 5 subgroups within the NADESs, which are as follows: ionic liquids (acid + base), neutral (sugar + polyols), acidic neutral (sugar/polyols + organic acids), alkaline neutral (sugar/polyols + organic bases), and amino acid containing (amino acid + sugar/organic acids). For the acidic neutral- and the amino acid-containing NADESs it has been observed that all components involved acts as both HBA and HBD. Meaning all molecules are arranged through hydrogen bonding and other intermolecular forces, acting like liquid crystals (Dai et al., 2013).

Since the NADESs components are strictly primary metabolites, it has made some explore the idea that they might occur naturally, as the required components will be abundant in cells. Some speculations regarding which role they might serve in cells has centred around assisting solubilisation and transportation of poorly water-soluble metabolites, storage, and adjusting the water content. During a study by Dai et al (2021), plant materials and secretions were analysed, and high levels of NADES components were found in the cells, often in optimal molar ratios for making a eutectic system. By looking at plants that has experiences drought, they found that NADESs have formed in the cells as the water evaporated. These NADESs would also stay liquid at low temperatures suggesting that they may play part in cold resistance as well (Dai et al., 2013). The evidence found strongly supports the theory of NADESs being present in plants, but it still needs further studying to conclude confidently.

2.2.2.1 Environmental aspects

Compared to DESs, NADESs have an environmental advantage which makes it more attractive to develop for future processes. Meeting the crisis of environmental ruin, humans are trying to adapt a greener direction in their way of life. For the extraction of collagen, the desire is to reduce the risks and hazards associated with the production process, and its impact on human health and the environment. NADES has so far been considered non-toxic, environmentally sustainable and biodegradable, reason being that they are made from primary metabolites. During a study by (Radošević et al., 2015) choline chloride – based NADESs were assessed for their biodegradability. It was found that all the formulations were readily biodegradable, showing high levels of mineralisation. Based on this, NADESs could be regarded as green solvents.

There is a possibility of classifying NADESs as food grade as well, but even if they are of primary metabolites, that does not mean they are suitable for implementation in the manufacture of products

meant for human consumption. For this, it would become necessary to choose NADES components specifically for their safety, and then it would need to be officially tested and approved. NADES must at some point become regulated by the Food and Drug Administration (FDA), Food and Agriculture Organization (FAO), or the European legislation. For instance, choline salts and their esters, and urea reaction products are on the EU prohibited substances list for cosmetic products, which would make extraction products from such NADESs difficult to use for cosmetic applications (ER No. 1223/2009 of the European Parliament and of the Council on Cosmetic Products). A limitation to NADESs being approved and regulated is due to the lack of research on which impacts and effects NADESs might have on human health, which makes it crucial that the science community continues to explore the topic. Some studies have in fact shown that the combination of the compounds in the NADESs are more toxic than the individual compounds, which are regarded as safe (Hayyan et al., 2013). In this specific study by Hayyan et al (2013), the toxicity and cytotoxicity of a few choline chloride- and phosphonium-based NADESs formulations on bacteria were evaluated. Regarding both toxicity and cytotoxicity, it was clear that the NADESs were more toxic than the constituting components themselves, and phosphonium-based ones were quite a lot more toxic to the bacterial strains compared to choline chloride-based ones. It is known that the delocalised charges in hydrogen bonding is more toxic than localised charges, which how they explain the observation. Radošević et al (2015) explored the toxic effect choline chloride – based DES has on fish and human cells. Most of the DESs did show some degree of cytotoxicity, and the choline chloride : oxalic acid formulation was moderately toxic. It is therefore way too early to decide if NADESs can be food grade or generally regarded safe. Each NADES that is to be considered for the application in a production process should be evaluated on their toxicity as it may vary with each formulation, and further studies are needed to gain a better understanding of their impact on organisms and environment

2.2.3 NADES for the extraction of collagen

Collagen as already been extracted using NADESs as solvents. For instance, Bai et al (2017), used, among other formulations, a choline chloride : oxalic acid (ChCl-OA) formulation at a 1:1 molar ratio to extract collagen from cod skins. ChCl-OA was reportedly the most effective formulation, and with it also being the most acidic, they concluded that acidity affects the extraction efficacy. They argued that because oxalic acid increases the number of free hydrogen protons, it resulted in binding sites between the protons and the secondary amine of proline / hydroxyproline in collagen, causing solubilisation. Using higher temperatures resulted in an observed increase of extraction efficacy to > 90% at 75°C, and a longer extraction time gave the highest purity at > 95% after 7 hours. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was also performed on the collagen product, which

seemed to contain peptides below 11 kDa while the other NADESs still showed clear α- and β- collagen chains. This implies that the ChCl-OA formulation could somehow create collagen peptides, meaning the solvent might have proteolytic characteristics.

In a subsequent study, Bisht et al (2021) tested different NADES formulations for extraction of collagen type I from Atlantic cod, in which the urea : lactic acid at a 1:2 molar ratio was the best. The NADES itself also performed markedly better at extraction than the constituent components would. SDS-PAGE showed the extracted collagen included both α1 and α2 collagen chains, which are the main constituents of type I collagen, meaning the NADES was able to extract the collagen without disrupting its structure. To support this finding, circular dichroism showed that the extracted collagen possessed a triple helix structure, with some random coil structure, indicating that the collagen was not denatured.

Furthermore, Grønlien et al (2020) formulated an acidic – neutral NADES for the solubilisation of collagen. They explored the possibility of NADESs, and collagen being utilised in combination for wound dressing, where collagen would promote wound healing, and the NADES would act as an antibacterial. The formulation of the NADESs was citric acid : xylitol : water in a 1:1:x ratio, where different molar ratios of water was assessed. They dissolved 3 mg/mL collagen peptides in the NADESs, and freeze dried the mixtures to sheets as potential wound dressings. The result was sheet-like sponges of varying plasticity, where some were easier to handle than the others. SDS-PAGE analysis of the mixture showed presence of both collagen type I and III, meaning the solvent did not damage the collagen molecules.

Batista et al (2022) used the same formulation as Grønlien et al (2020) for the extraction of collagen from blue shark skin waste. Here, the citric acid : xylitol : water mixture was at a 1:1:10 ratio and synthesised by the heating method. The collagen yield was 18.6%, which is comparably higher than the conventional method which yielded 7.6%. They do speculate that this could also be due to the temperature difference at which the processes were done, 4°C during conventional, and 40°C during the NADES facilitated extraction. The extracted collagen was dissolved in acetic acid to make a protein extract. Protein content of extract was shown to be > 80%, and SDS PAGE showed that the sample was of quite pure collagen type I.

2.3 SUBSTANCES USED IN THE PRESENT STUDY

The aim of the present study was to explore how NADESs can be utilized for the solubilisation of collagen in an extraction process, and to see what kind of properties they have as solvents. With a desire to gain a better understanding of exactly how the solvents interact with collagen, gelatin and cellulose were chosen as representative samples.

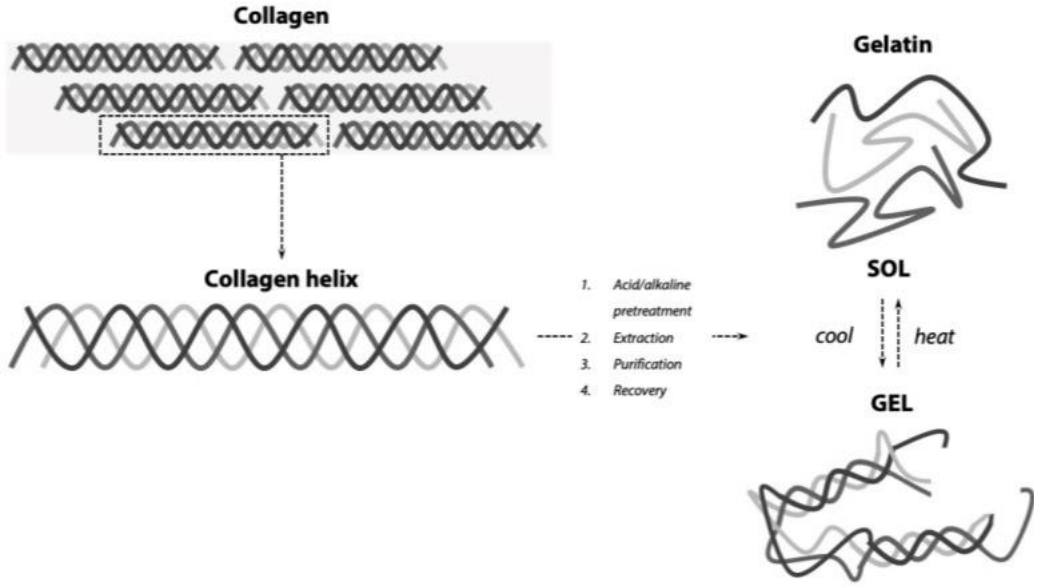


Figure 3: The diagram shows the process of collagen being hydrolysed in an extraction process, producing gelatin as the product. Gelatin in a heated solution will be solubilised, and as the solution cools, triple helices will reform through hydrogen bonding, forming a network of peptides. The trapping of water within this structure forms a gel. (From: (Campiglio et al., 2019)*)*

As mentioned earlier, gelatin is the product of collagen being partially hydrolysed. Even if the collagen structure is hydrolysed, the peptide chain remains the same. Gelatin is therefore used as a sample to represent the primary structure of collagen. Depending on its source and to which purpose it is extracted, gelatin can have a wide range of viscosity and bloom values. Each collagen molecule in the triple helix structure is approximately 95 kDa, two of them being type α1, and the last one being type α2. Because the collagen is partially hydrolysed the molecular weight might vary a lot between peptides depending on how bound together the molecules still are (Keenan, 2012). An important property of gelatin is the ability to form gels. Gelatin is highly hydrophilic and will in a solution act as a random coil under the influence of heat. Gels will form when the solution is cooled to < 35°C, but above this temperature, gelatin is incapable of forming interchain H-bonds (Deshmukh et al., 2017). As the gel forms helical regions will form hydrogen bond associations both inter- and intramolecularly with other collagen molecules, reforming the triple helices, while other parts stay as random coils, as illustrated in Figure 3. As a result, a network is formed which traps water within the structure, creating a hydrogel (Keenan, 2012; Van Vlierberghe et al., 2014).

With gelatin representing the amino acid sequence of collagen, cellulose represents its macrofibre structure. Cellulose is a polymer of β-1,4-anhydroglucopyranose with typically 300-1000 units in each molecule. The formation of microfibrils from cellulose chains is a spontaneous process, as intramolecular hydrogen bonding forms between parallel planar molecules (Shanks, 2014). The cellulose polymer chains will constitute a cellulose fibre similar to collagen fibres as they bundle up, shown in Figure 5. The microfibrils consist of crystalline and amorphous regions, and it is the crystalline regions that are the constituents of cellulose nanocrystals (Gumrah Dumanli, 2017). Due to this highly crystalline structure, and its inter- and intramolecular hydrogen bonding, cellulose is quite insoluble in water, similarly to collagen. According to Naz et al (2016), hydrolysis of cellulose will happen at low pH, making it swell with water and become soluble. An increase of temperature would promote this further. They also noted that the solubilised cellulose had a milky appearance, meaning that it is more like a colloidal solution than a true solution. This could potentially make it difficult to assess certainly whether the cellulose sample dissolved in the solvent, or not.

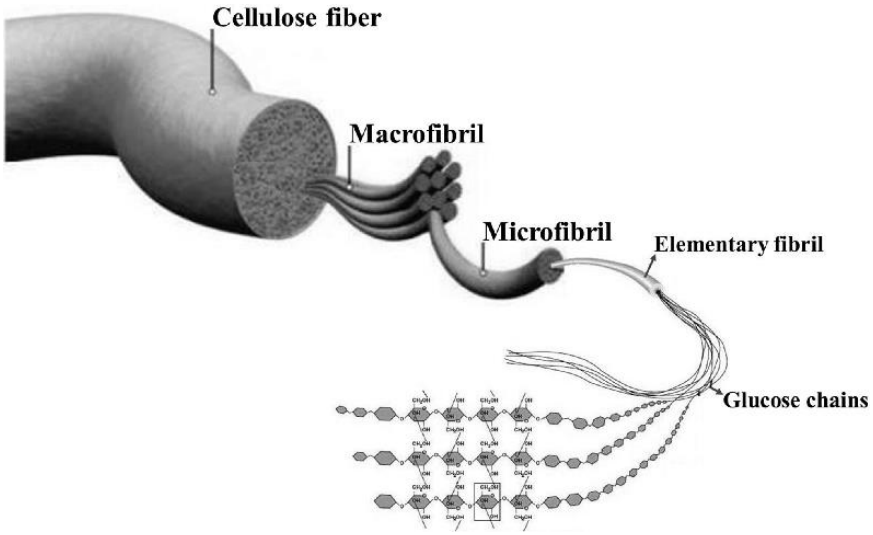


Figure 5: Long glucose chains interact together through hydrogen bonding forming fibrils on many hierarchical levels until a cellulose fibre is formed from these subunits. (From: (Muthuraj et al., 2018)*)*

NADESs have been extensively applied for dissolving cellulose. For instance, Ling et al (2019) used a choline chloride : oxalic acid dihydrate (ChCl-OAD) system to extract and assess cellulose nanocrystals. For this, one gram bleached cotton was added to 100mL NADES and stirred for 2 hours at 80-100°C. As a result, they observed a 10-20% dissolution of the cellulose sample. Nevertheless, the researchers also recognised that the extraction was performed at a very high temperature, meaning that it could have cause excessive breaking of the hydrogen bonding in cellulose. Similarly, Sirviö et al (2016) experienced a 33% dissolution of cellulose pulp from wood when using the same NADES formulation. The treatment here was also performed at 100°C, meaning that could again give a heightened effect. Douard et al (2021) also utilised this ChCl-OAD system at a 1:1 ratio for extraction of cellulose

nanocrystals. The best conditions were found to be at 95°C, resulting in a cellulose nanocrystal yield of approximately 44.9%.

The aim of the present study was to prepare NADESs of different formulations, and to assess their solubilising efficacy on gelatin and cellulose in comparison to water. The information gathered in the study can potentially be exploited for developing a process that enables the extraction of collagen from fish biomass. By mainly focusing on the characteristics of the NADESs as solvents, there is a hope to gain a better understanding around exactly how they work, what their properties are, and if the application itself is feasible in reality. During a preliminary testing, many NADESs were tested and two were chosen for the main study. The first one was the CA-X-W formulation by Batista et al (2022), and the second was the ChCl-OAD formulation by Ling et al (2019). Both were applied to the gelatin and cellulose samples in a solubilisation assessment. The supernatant, as well as the solvents themselves were subjected to a series of analyses to gain a clearer view of the bigger picture.

3 MATERIALS AND METHODS

The experiment design is shown in Figure 6 below and goes over all the major steps of the project, as well as all the performed assays. The project started with preliminary testing of six different NADES formulations to determine which were the most suited for potential collagen extraction. The most suitable NADESs were remade, and solubilisation of the sample materials was performed. The precipitate was dried to calculate yield, and the supernatants were analysed as shown in the figure.

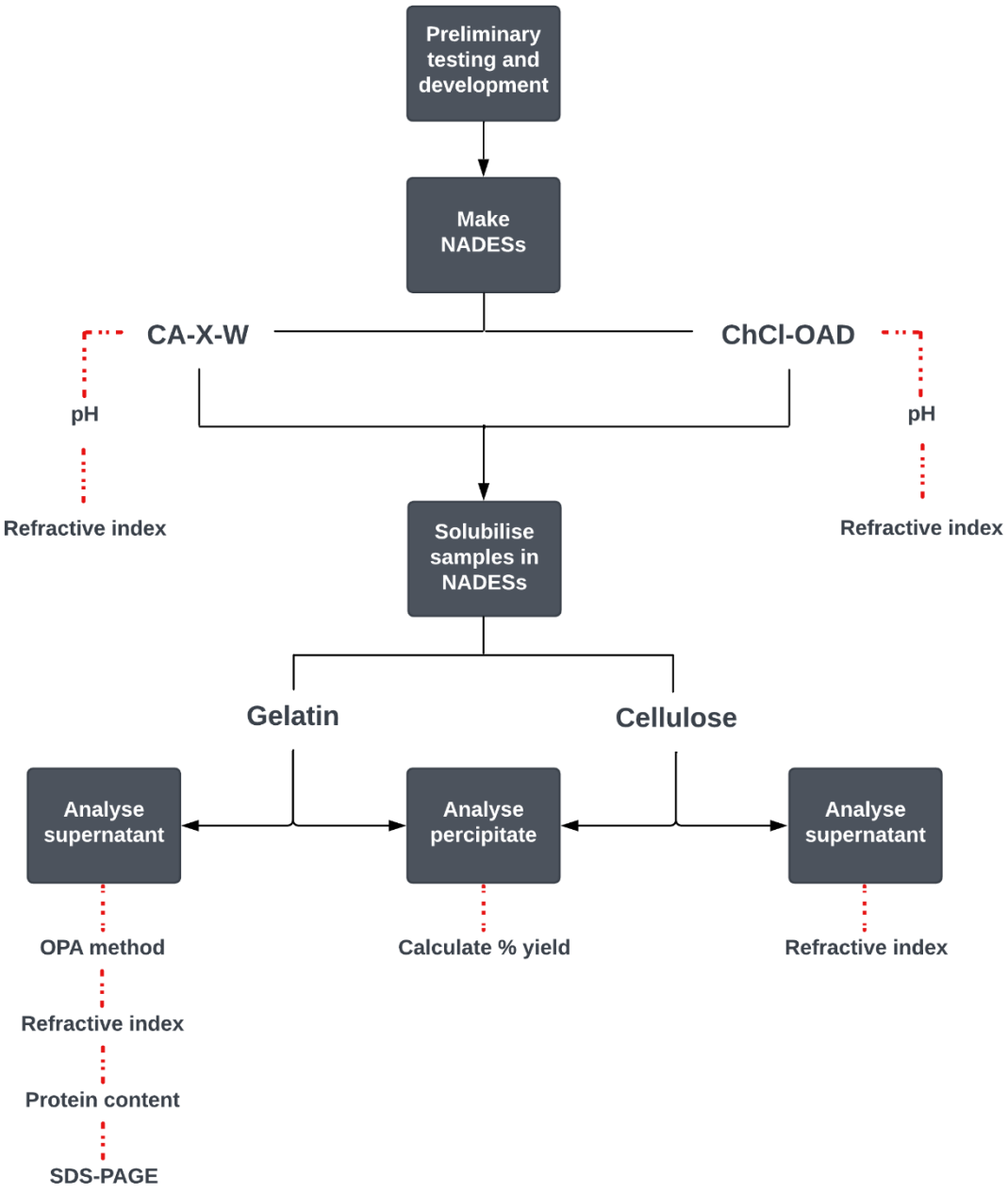


Figure 6: Flowchart of the main moments of the project. Dotted lines indicate an analysis which was performed on the sample the line originates from.

3.1 MAKING NADES

Chemicals

- Choline chloride (CAS-No: 67-48-1, obtained from Sigma-Aldrich)
- Oxalic acid dihydride (CAS-No: 6153-56-6, obtained from Sigma-Aldrich)
- Oxalic acid (CAS-No: 144-62-7, obtained from Sigma-Aldrich)
- Urea (CAS-No: 57-13-6, obtained from Sigma-Aldrich)
- L(+)-Lactic acid (CAS-No: 79-33-4, obtained from Sigma-Aldrich)
- Citric acid (CAS-No: 77-92-9, obtained from VWR International AS)
- Xylitol (CAS-No: 87-99-0, obtained from Sigma-Aldrich)
- DL-Malic acid (CAS-No: 6915-15-7, obtained from Sigma-Aldrich)

As part of the preliminary testing, six NADES formulations were made and briefly assessed. An overview of all formulations is shown in Table 1, stating the molar ratio of the components, which component is HBA, and which is HBD. Ahead of making the solvents, choline chloride was dried at 105°C for 24 hours. All the components were then weighed to the correct molar ratio and mixed by light manual shaking in 250 mL Erlenmeyer flaks (E.g., a 1:1 ratio of ChCl-OAD would equate mixing 69.81 grams choline chloride with 63.03 grams oxalic acid dihydrate to make a 132.84-gram batch of solvent).

*Table 1: Solvent names are abbreviations of the constituent components. Additional information shown is the molar ratio at which they are mixed and which component is HBA and HBD. Any component marked with * can act as both HBA and HBD*

Solvent	Molar ratio	HBA	HBD	Third component
ChCl-OAD	1:1	Choline chloride	Oxalic acid dihydrate	
ChCl-OA	1:1	Choline chloride	Oxalic acid	
U-LA	1:2	Urea	L(+)-Lactic acid	
CA-X-W	1:1:10	Citric acid*	Xylitol*	Water (HBD)
ChCl-X-MA	1:1:1	Choline chloride	Xylitol*	DL-Malic acid*
MA-X	1:1	Malic acid*	Xylitol*	

A magnetic stirrer was added in each flask and the top was covered with aluminium foil and secured with tape. Using a magnetic stirrer plate with heating, a large beaker with distilled water was heated to 60°C. A thermometer was used to ensure that the temperature stayed consistent throughout the procedure. The flasks with NADES components were placed and secured in each water bath and stirred until a homogenous and translucent liquid was obtained. Some solvents required a modified procedure to reach liquification. The temperature for ChCl-X-MA was after some time increased to 70°C, and the MA-X formulation was transferred to a round bottom flask and connected to a rotary evaporator (RV8,

IKA, Staufen, Germany) where the temperature was slowly increased to 95°C. Marked 50 mL tubes were used to store the NADESs until use.

The last solvent being double distilled water was tapped directly from the tank right before use.

3.2 SOLUBILISING SAMPLES

Materials

- Gelatin from porcine skin, Type A (CAS-No: 9000-70-8, obtained from Sigma-Aldrich)
- Cellulose filter papers (Whatman® qualitative filter paper, Grade 1, 55mm)

The NADESs which were successfully synthesised, were also tried for a preliminary solubilisation of gelatin and cellulose as an initial assessment. 0.2 grams of gelatin and cellulose filter paper were weighed into 50 mL tubes, only one replicate was taken for each solvent with each sample. The filter papers were cut into small pieces before being placed in the tubes. To each sample, 19.8 grams of solvent was added to make the dilution 1% w/w. A magnet was added to each NADES as well to assist in mixing. They were all incubated horizontally in a shaking incubator (Minitron, Infors HT, Bottmingen, Switzerland) at 40°C for 4 hours, with a speed of 340 rpm (1,61 rcf).

After the preliminary testing, the NADES formulations were narrowed down to two, which would be thoroughly assessed for the solubilisation of gelatin and cellulose. Based on preliminary results the conditions were adjusted and the solubilisation redone. The new conditions for the gelatin sample were 7% w/w concentration, at 25°C for 4 hours, with the speed remaining at 340 rpm. This equating to 1.4 grams gelatin in 18.6 grams solvent. While for cellulose the conditions were same except for the incubation time being increased from 4 hours to 24 hours. The 50 mL tubes were marked and weighed before the samples were added. All weights were accurately noted down.

The new conditions were approved and now three replicates would be taken rather than just one. This was first done with gelatin, and to save materials the sample sizes were scaled down to half while the conditions remained the same. Using 15 mL tubes, 0.7 grams gelatin was weighed out and added to 9.3 grams of solvent and incubated at 25°C for 4 hours. This method was immediately rejected and not performed on cellulose.

For the final solubilisation the amounts were scaled back up to the total of 20 grams using the 50 mL tubes. Three replicates were taken of both gelatin and cellulose in each solvent, and extra accuracy in weighing was implemented to ensure precision. The weights of empty tubes without caps, samples materials as well as solvents were accurately noted down throughout the process.

After being incubated, the tubes were centrifuged using a refrigerated centrifuge (Multifuge X1R, Thermo Scientific, Osterode, Germany) at 5500 rpm (approximately 5073 rcf), at 20°C for 20 minutes. All supernatants were then separated out by pipetting, leaving behind the precipitate and some solvent, and loaded into 15 mL tubes for storage.

For every part of the solubilisation, distilled water was used as a control solvent for both samples, having the same conditions as described throughout.

3.2.1 Solubilisation yield

A couple of methods for measurement of the solubilisation yield was tested out. After centrifugation, the supernatants were removed, and distilled water was added into all the tubes. The sample was shaken using a vortex mixer, and then the mixture was poured into a funnel containing a pre-weighed glass filter paper to catch the undissolved sample particles. The tube was washed with more water a few times until all particles were emptied into the filter. Afterwards, the filters would then be dried and weighed. This method was rejected.

The chosen method for calculating the yield starts the same by adding distilled water back into the tubes and mixing sufficiently. The tubes were centrifuged at 5500 rpm (approximately 5073 rcf) at 20°C for 20 minutes. The supernatant was carefully removed by decantation, and more water was added before another round of centrifugation. This washing process was repeated a total of 5 times. After the last centrifugation the water was carefully removed by decantation, and the tubes were placed in a drying oven at 105°C for > 24 hours without caps (McDonald et al., n.d.). The fully dried samples and tubes were then weighed to calculate the yield for how much was solubilised. Calculations were done using Equation 1.

Equation 1: Yield of solubilisation was calculated as the complementary ratio of the weight of dried precipitate to the weight of the original sample

$$Yield\ (\%) = 1 - \frac{Weight\ of\ dried\ percipitate}{Weight\ of\ original\ sample} \times 100$$

3.2.2 pH of solvents

A sufficient amount of each solvent was poured into small 10 mL beakers. The pH was measured using a benchtop pH meter (accumet™ AE150, Fisherbrand™, Osterode, Germany). The electrode was thoroughly cleaned after each solvent, and proper removal was ensured by doing a control measure

of distilled water between the measurement. There was only done one replicate of each solvent. The measurement was taken as the pH had stabilised for at least 5 seconds.

3.3 Refractive index

The refractive index was measured using a benchtop refractometer (RX-5000, Atago, Tokyo, Japan). All supernatants, from both gelatin and cellulose solubilisation were measured, as well as the solvents themselves, three replicates of each. To do this, a few drops were pipetted onto the prism seat of the refractometer. The cover was then closed, and the sample was read. After each reading, the prism seat was thoroughly cleaned with soft lens cleaning tissues and distilled water.

3.4 Assessing supernatants with solubilised gelatin

The following assays were only performed on the solvent supernatants from the solubilisation of gelatin samples.

3.4.1 Concentration of primary amino groups

Chemicals

- Phthaldialdehyde Reagent "OPA" (CAS-No: 643-79-8, obtained from Sigma-Aldrich)
- Glycine (CAS-No: 56-40-6, obtained from Sigma-Aldrich)

Primary amino group concentration was found by performing an o-pthalaldehyde (OPA) assay. The method is followed as described in the first protocol by Interchim. An extract of the method can be found in Appendix 1. Step 1 and 2 is overlooked as OPA reagent is bought as a complete solution, and not made from scratch. The standard curve was made using glycine, with dilutions ranging from 0.1 mM to 1 mM.

100 µL of each supernatant from gelatin solubilisation and the glycine standard was pipetted into semi micro cuvettes. Three replicates were taken of each supernatant and standard, and the blank was made with 100 µL water. 1 mL OPA reagent was then added to the blank, standards and samples, and mixed in thoroughly. Using a UV-Vis Spectrophotometer (10S, Thermo Genesys, Madison, USA), the blank is set at 340 nm. All samples, standards and the blank were read at exactly 2 minutes after the OPA reagent was added.

The concentration of primary amino acids in the supernatants was then calculated using the plotted standard curve of glycine.

3.4.2 Protein content

Materials

- Bio-Rad protein assay dye reagent concentrate (obtained from Bio-Rad Norway AS)
- Bovine serum albumin (CAS-No: 9048-46-8, obtained from Sigma-Aldrich)

The determination of total protein content of the gelatin supernatants was done by the Bio-Rad method (Nobel, 2000). This is a simple rapid test using the principle of the Bradford protein-dye binding method.

The standard was prepared by making a 1.5 mg/mL solution of bovine gamma globulin, and then diluting it into a series ranging from 0.3 mg/mL to 1.5 mg/mL. The dye reagent was prepared by mixing 1 part concentrate with 4 parts distilled water. Supernatants were suitably diluted, and water was used as blank. 100 µm of each supernatant, standard, and the blank were pipetted into clean test tubes. 5 mL of the diluted dye solution was added and mixed in well. Three replicates were taken of each supernatant and standard. The readings were done on a UV-Vis Spectrophotometer (10S, Thermo Genesys, Madison, USA) at 595 nm after 5 minutes, making sure to mix right before the measurement.

3.4.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Chemicals

- Dithiotreito “DTT” (CAS-No: 3483-12-3, obtained from Sigma-Aldrich)
- NuPAGE™ LDS Sample Buffer (4X)
- Glycerol 10%
- PageRuler Broad Range Protein Ladder
- NuPAGE™ 4-12% Bis-Tris Gel
- NuPAGE™ MOPS SDS Running Buffer (20X)
- InstantBlue protein stain
- Destaining solution (30% methanol, 10% acetic acid, 60% distilled water)

SDS-PAGE was performed to obtain knowledge about the peptide size distribution in the supernatants from gelatin solubilisation. Using the results from the protein content assay, the supernatants were diluted to 0.2-0.4 mg/mL. One replicate of each supernatant was done. To prepare the buffer, 0.0124

grams DTT was weighed into a 0.5 mL Eppendorf tube and dissolved in 200 µL LDS sample buffer. In 0.5 mL Eppendorf tubes, 30 µL sample was mixed with 10 µL buffer, and placed in a water bath at 70°C for 10 minutes. 4 µL 10% glycerol was lastly added to the samples. The pre-cast SDS gel was rinsed and placed in the chamber, and the inner and outer chamber was filled with buffer diluted 1:20. 10 µL of the samples and the protein ladder was loaded into the wells as shown in Table 2 below. The gel was then run at 200 V for around 50 minutes. The gel was removed from the cassette and stained with InstantBlue protein stain to colour the bands. The destaining process was done manually on an orbital shaker with destaining solution until a satisfactory amount of stain had been removed.

Table 2: ChClx represents the ChCl-OAD supernatants, CAx represents the CA-X-W supernatants and Wx represents the supernatants from water. Well 2 and 3 contains the respective NADESs without any sample dissolved in them.

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Ladder	ChCl-OAD	CA-X-W	ChCl1	CA1	W1	ChCl2	CA2	W2	ChCl3	CA3	W3

4 RESULTS AND DISCUSSION

4.1 FORMULATION OF NADESS

All the NADESs prepared in section 3.1 were assessed for their ease of preparation and handling, as well as their solubilising capabilities. The results are discussed below, and a full overview of their traits and specifications are reported in Table 3.

Citric acid : Xylitol : Water (CA-X-W)

This formulation was first made for collagen by Grønlien et al (2020) as an excipient for collagen-based products and was later applied by Batista et al (2022) for the extraction of collagen from blue shark skins. The collagen extraction yield by NADESs was 18.6%, which compared to the conventional extraction method by acid, is roughly 2.5 times higher (Batista et al., 2022). The formulation was chosen for the preliminary testing of NADESs based on those results. The formulation was a very quick and efficient to synthesise, taking less than 30 minutes at 60°C. The heat could be adjusted lower as well, which would make the synthetisation more energy saving. Additionally, it was observed to have a very low viscosity, which is of benefit for potential extraction processes. All solvent components are food grade, making it a good candidate for applications meant for human consumption, though the solvent should be thoroughly tested for toxicity. When this solvent went through preliminary testing for solubilisation of 1% w/w gelatin at 40°C, there was no visible sample left after drying. Based on all these results, the solvent was deemed a good candidate for the solubilisation of collagen and was chosen for further testing.

Table 3: An overview of all the formulations that were tried and their individual traits. "solubilisation" refers to their observed ability to dissolve gelatin at 1% w/w at 40 degrees

Formulation	Molar ratio	Able to make	Short preparation time	Low viscosity	Food grade	Solubilisation	Chosen
CA-X-W	1:1:10	✓	✓	✓	✓	All	✓
U-LA	1:2	✗	-	-	✗	-	✗
ChCl-OAD	1:1	✓	✓	✓	✗	Almost all of it	✓
ChCl-OA	1:1	✓	✓	✗	✗	A bit	✗
MA-X	1:1	✓	✗	✗	✓	No	✗
ChCl-X-MA	1:1:1	✓	✗	✗	✗	No	✗

Urea : L(+)-Lactic acid (U-LA)

This next formulation was used by Bisht et al (2021) for the extraction of collagen from cod skin. They obtained a collagen yield at around 6% of fish sample and was therefore chosen for preliminary testing. During the synthetisation process, the components were weighed and mixed, but no melting occurred. There was observed a white chemical escaping out of the flask, which was suspected to be lactic acid.

A couple of observations were made regarding this issue. Their method describes using a round bottom flask and a rotary evaporator, doing this would prevent the chemical from escaping. Also, the article states using aqueous solutions of U-LA, not pure U-LA. This was not taken notice of until after preliminary testing. The addition of water was not mentioned in the "DES preparation" section either, so it is not clear when it should have been added, or how much. Using this information, the solvent could be tried again, but had already been disqualified from further use as it contains urea, a material which should not be used in consumption products (Dickerson et al., 2018).

Choline chloride : Oxalic acid dihydrate (ChCl-OAD)

This formulation has been utilised for the extraction of cellulose nanocrystals and has shown promising results. Douard et al (2021) extracted 44.9% of nanocrystals from cotton, while (Ling et al., 2019) experiences 10-20% dissolution of their samples. Additionally, Sirviö et al (2016) also experienced positive results for cellulose. This formulation was chosen for preliminary testing wating to explore if the same effect would be expressed on the cellulose samples here, and if anything would happen to gelatin as well. The synthetisation was determined to take less than an hour and could probably have been made at a lower temperature than 60°C. Another advantage of this solvent was its low viscosity, making it good for extraction processes, as well as being able to dissolve almost all the gelatin under the testing with 1% w/w at 40°C. However, this solvent has choline chloride as a component, which might cause limitations in some applications. As stated earlier, choline salt and esters are prohibited by the EU for use in cosmetic products (ER No. 1223/2009 of the European Parliament and of the Council on Cosmetic Products), making this a possible limitation for use in such products. It is, however, still of interest to test this solvent since the extraction product could have other applications, such as food. The upper limit for intake choline a day is 3.5 grams for adults (*Choline - Fact Sheet for Health Professionals*, 2022), so assuming the solvent is well separated from the extraction material, it might someday be approved for use. Considering the solvent's decent solubilisation of the gelatin and low viscosity, it was chosen for further testing.

Choline chloride : oxalic acid (ChCl-OA)

This formulation was used by Bai et al (2017), and chosen for preliminary testing as they were able to extract collagen peptides from cod fish skins. They stated the collagen was proteolysed to smaller peptides by the solvent. If this is correct, it would be interesting to do further research to explore if these peptides can have different applications such as bioactive compounds. However, when using this solvent during preliminary testing, a few conflicting observations were made. For starters, the solvent was relatively quick to make, but had a very thick and viscous character, and only a small amount of gelatin was dissolved during preliminary testing. It was also noted by Radošević et al (2015) that this formulation had a moderately toxic effect on fish and human cells. Based on these results, ChCl-OA

was not chosen to go further in this study. Additionally, the results are conflicting with those of Bai et al (2017) stating they had an efficacious extraction. There might be an explanation to the deviation in our results. They measured the viscosity of the ChCl-OA solvent to be 156 mPas, which is very close to the that of tomato juice at around 180 mPas (Mirondo & Barringer, 2015). Seeing as the solvent should be less viscous than a fruit juice, it is quite evident that the solvent which was synthesised for this study is not the same as the ChCl-OA they made. The hypothesis, therefore, is that they used oxalic acid dihydrate in the formulation (ChCl-OAD), not oxalic acid, as the former had a much more suitable viscosity for dissolving samples when made for the preliminary testing. This cannot be known for sure, but further testing using ChCl-OAD will show if the results are similar to those stated by Bai et al (2017). It could also be possible that they did use oxalic acid, but the chemical could have been hydrated already before use, due to air moisture during storage (Lorenzetti et al., 2022).

DL-Malic acid : xylitol (MA-X) and Choline chloride : Xylitol : DL-Malic acid (ChCl-X-MA)

Both solvents were chosen from Table 1 in the article by Dai et al (2013), which explores the use of NADESs as a green technology. They were not applied to any solubilisations or other applications in their work, it was simply stated that both are eutectic mixtures. Based on the literature, it seems that most solvents tried on collagen are of the “acidic neutral” or “ionic liquid” type of NADES. These two solvents were therefore chosen for preliminary testing since they have a similar characteristic. Both solvents took very long to synthesise, and the viscosity was considerably high. For the MA-X formulation, the components were after a while transferred to a round bottom flaks and connected to a rotary evaporator to assist with stirring. The temperature had to be gradually increased to 95°C before it reached a homogeneous liquid. ChCl-X-MA also took a while to make, but was a bit quicker and less viscous than MA-X. The temperature had to be increased to 70°C for the components to melt into a homogenous liquid. Since the formulation of this solvent is the same as MA-X, but with the addition of choline chloride, it would seem this extra compound might be the reason for the slightly lower preparation time and temperature. Adding water to the formulations might also reduce their viscosity, but considering the scope of the study, these solvents were discarded as they were.

The only two solvents which solubilised a considerable amount of gelatin was ChCl-OAD, having dissolved almost everything, and CA-X-W, having no visible sample left. Distilled water was always used alongside the solvents as a point of reference, and it had also no visible gelatin sample left at 1% w/w and 40°C after 4 hours. To confirm the finding, and to find out the exact solubilisation efficacy, the conditions were adjusted. From then on, the gelatin was used at a concentration of 7% w/w, and the temperature was reduced to 25°C. These adjustments would make it more difficult for the solvents to dissolve everything, especially water, making it easier to compare them.

As mentioned in section 3.2, there was a downscaling from 20 total grams to 10 grams. This was done in 15 mL tubes, with the reason being to save materials. By doing so, the solvents and samples would fill up to the top of the tube, rather than only halfway, like it did in 50 mL tubes. This, coupled with the narrowness of the tubes, resulted in the flow and movement of the solvents being highly restricted during shaking. Resulting in the solvents being a lot less effective. On this basis, the downscaling was rejected, and the original amount of 20 grams in 50 mL tubes was reinstated as the preferred method for solubilisation.

Except for MA-X, which was placed in a round bottom flask, all other solvents were stirred using a magnetic stirrer. This proved to be a mistake as the magnetic force was too weak to create proper mixing in the hard-to-melt eutectic mixtures. The main cause of this difficulty is that the mixture becomes very sticky as some sections begin melt, creating a very lumpy mass. To solve this issue, it is recommended to use a rotary evaporator instead, avoiding the use of magnets all together.

If doing this same procedure later, there are a few more comments which are worth noting. Most components of NADESs, like choline chloride, have high affiliations with water, meaning they could contain a lot of moisture ahead of making the solvents (Lorenzetti et al., 2022). Therefore, it is recommended to dry all chemical components at 105°C for 24 hours before use to ensure no additional water is present in the solvent unaccounted for. Additionally, the NADESs should be synthesised under a closed environment, like with the rotary evaporator using round bottom flasks as described by Batista et al (2022). This will further ensure that the NADES has the desired water content at the end.

4.2 ASSESSMENT OF THE SOLUBILISATION EFFICACY

4.2.1 Gelatin

Figure 7 shows overview of the average solubilisation efficacy of the solvents stating the percentage of gelatin dissolved at 25°C after 4 hours of shaking. Standard deviations can be found in Appendix 2. An analysis of variance showed the p-value to be is less than 0.05, meaning that there is significant difference between the solubilisation efficacies.

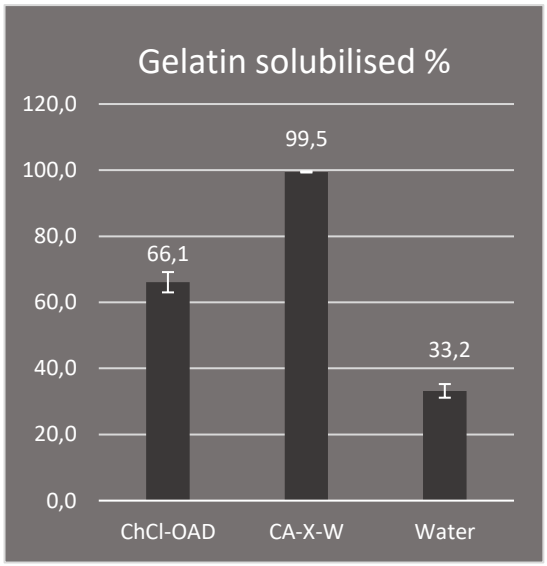


Figure 7: The choline chloride : oxalic acid dihydrate (ChCl-OAD) solvent solubilised 66.1% of the gelatin sample, while citric acid : xylitol : water (CA-X-W) solubilised 99.5%, and water solubilised 33.2%.

66.1% dissolved gelatin is equivalent to 0.925 grams of the original 1.4-gram gelatin sample. This makes ChCl-OAD on average a better solvent than water at these conditions, but not as good as CA-X-W. As mentioned in section 4.1, it is suspected that Bai et al (2017) was using ChCl-OAD (oxalic acid dihydrate) in their experiments rather than ChCl-OA (oxalic acid), as they stated in their paper. Considering the high solubilising efficacy of gelatin for ChCl-OAD, this suspicion seems to be accurate as they also experienced considerable results for collagen solubilisation. During their study, collagen was extracted from cod skins with a yield close to 45% of the available collagen, and they explained the solubilising effect to be due to the acidity of oxalic acid. Many factors can explain the difference in results between their study and the ones shown here. Firstly, they used dried cod skins, which contain other constituents than just collagen. This reduced bioavailability would be a limitation as to how much collagen could be extracted, compared to the current study, where a pure material was dissolved. Secondly, the material used in this study was gelatin, which is the hydrolysed version of collagen. The process of hydrolysing native collagen to gelatin causes a destruction of the fibrous structure, resulting in the availability to each collagen molecule being much higher than it is in a fish skin matrix. This as well would make it a lot easier to dissolve the gelatin, potentially explaining why there is such a significant difference in yield.

CA-X-W, being the best solvent out of all three, dissolved on average 99.5% of the gelatin, which is equivalent to 1.392 grams of the original sample of 1.4 grams. This solvent was used by Batista et al (2022) for the extraction of collagen from blue shark skins. They obtained an extraction yield at 18.6%

of the shark skin sample. This might be a very good result, but it is difficult to determine without knowing the total amount of collagen in the sample, meaning 18.6% could be of the entire sample, or of the total available collagen. Therefore, it is difficult to compare these results with theirs, but as mentioned for ChCl-OAD, other constituent present and lower bioavailability would decrease the extraction efficacy compared to pure gelatin. Either way, considering over 99% of the gelatin got dissolved, it certainly has some properties which acts upon the structure. Grønlien et al (2020) also used the CA-X-W to dissolve collagen, and they theorised that the dissolving effect could be due to collagen diffusing to the liquid media as a response to the decrease in pH caused by citric acid. Furthermore, xylitol, and other polyols, are found to contribute as a stabilising component in native collagen. However, the xylitol did not express this effect as part of the solvent in their study. Either way, this solvent obtained a high yield of dissolved gelatin, and based on the literature, this effect should also be applicable to collagen.

Water was used as a point of reference for the solvents, and showed to be the least efficacious, dissolving on average only 33.2% of the gelatin, which is equivalent to 0.465 grams of the original sample of 1.4 grams. The purpose of it being a point of reference was to prove that ChCl-OAD and CA-X-W had a higher solubilisation efficacy on gelatin than water. If they did not, there would be little to indicate that they would work on collagen as well. What is interesting though, is that any gelatin dissolved at all, seeing as interchain H-bonds of gelatin should not break until around 35°C (Deshmukh et al., 2017), and the solubilisation was performed at 25°C. Gelatin in its undissolved form are present as triple helices, which will denature upon heating in an aqueous solution and revert to helices upon cooling. At which temperature this happens varies on the collagen composition (Gómez-Guillén et al., 2011). Gelatin does however hydrate at much lower temperatures, and some gelatin might start solubilising at lower temperatures, with 35°C being the point where everything should be dissolved. According to Sadowska et al (2003) is seems that gelatin dissolves at a temperature range, where some can dissolve at temperatures as low as 20°C. Based on this, a portion of the gelatin was able to dissolve in the water solvent, even if the temperature was only 25°C. However, it is not possible to state if the dissolved collagen molecules are representative of the whole sample, or if just a specific peptide size or type were dissolved.

It could also be a factor that the low temperatures for the solubilisation favoured NADESs to obtain a higher efficacy than water. If the conditions were at higher temperatures that 25°C, water might be a better solvent than ChCl-OAD, which was hinted at during the preliminary testing where water and CA-X-W appeared to perform better than ChCl-OAD at dissolving gelatin. This would have to be tested, as it is not clear to say what would have happened if only the gelatin concentration was increased, while the temperature stayed at 40°C, as that might give a better indication as to what the solubilisation

efficacy for gelatin is. However, collagen in its native structure is not water soluble, so the efficacy expressed here on gelatin would not be applied for actual collagen. Water was mainly used to confirm that the solvents had a considerable solubilisation efficacy of gelatin, since native collagen was not tested in this project. Both NADESs have however been tested for extraction of collagen from marine sources before, meaning it should be expected that they can be tested for a potential application in an extraction process.

This study was executed with the purpose of gathering some information which can be used to further explore the application of NADESs in the industrial extraction of collagen from fish biomass. When applying this to an industrial process, it would be natural to increase the temperature as that would also increase the extraction yield. However, collagen from cold-water fish will have a lower denaturation temperature than mammals as the composition has a lower proline and hydroxyproline content (Gilsenan, 2000). This is an aspect which should be further explored, so that this extraction method may be implemented as optimally as possible without unintentionally damaging the collagen.

4.2.2 Cellulose

The average solubilisation efficacy for cellulose is very similar between the solvents, as shown in Figure 8. Standard deviations can be found in Appendix 3. An analysis of variances showed the p-value to be more than 0.05, meaning there is no significant difference between the solubilisation efficacy of the solvents.

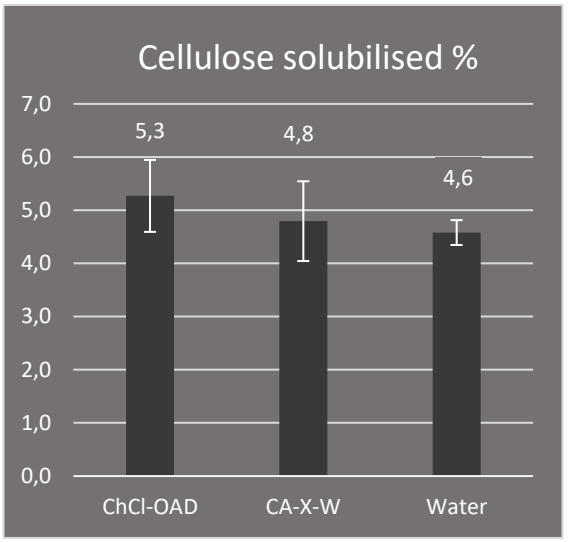


Figure 8: The choline chloride : oxalic acid dihydrate (ChCl-OAD) solvent solubilised 5.3%, while citric acid : xylitol : water (CA-X-W) solubilised 4.8%, and water solubilised 4.6%.

Cellulose is not water-soluble, and the solubilisation efficacy is expectedly quite low. Water is thus a very good reference point for comparison, since more suitable solvents should comparably have a more prominent effect on dissolving cellulose. Based on visual notes, the cellulose filter paper was completely hydrated with water after the solubilisation. The mass can be described as a homogeneous pulp with no visible pieces of paper left. There was also observed cellulose fibres floating in the supernatant after each centrifugation during the washing of the precipitate. This could explain the average result of 4.6% solubilised cellulose since the fibres floating in the supernatant would be removed during the decantation. The original cellulose sample

was 0.2, and 4.6% solubilisation is equivalent to 0.009 grams. Such a small loss could therefore have occurred by mistake during the washing.

The average solubilisation of cellulose by CA-X-W is close to that of water at 4.8%, and as mentioned, they are not statistically different. This solvent has only been used for the solubilisation of collagen and gelatin in the literature, meaning there was no expectation it would have any effect on cellulose as well. There was observed many similarities to water, such as a very hydrated cellulose pulp, and fibres were seen floating in the supernatant which was decanted off after centrifugation. Considering this formulation contains a high molar ratio of water, it could be thereof explained why it acted very similarly to the water solvent. Either way, the low solubilisation may suggest that this solvent might not have much effect on the collagen macro fibre structure itself, compared to its effect on the intermolecular forces of collagen molecules. However, this information is too vague to make any absolute statements.

Like the other two solvents, ChCl-OAD had a very low solubilising effect on cellulose, with only 5.3% solubilised on average, but according to the literature it should have been more effective. The difference might be due to the conditions for solubilisation being more extreme in the literature. Douard et al (2021) obtained a 44.9% yield of cellulose nanocrystals from cotton samples, but the extraction temperature was at 95°C. Similarly, Ling et al (2019) had a 10-20% dissolution of cotton cellulose at 80-100°C, and Sirviö et al (2016) had a 33% dissolution of cellulose pulp from wood when extracting at 100°C. Based on this, it would make sense there was no prominent dissolving activity at 40°C. However, based on visual notes, something might have happened to the cellulose structure, even if it was not to the same extent as these studies. Compared to CA-X-W and water, there was no floating fibres in the washing water supernatant after centrifugation, thus the 4.6% cellulose loss might be due to something else. The supernatant did however appear slightly milky in colour, meaning some cellulose might have been solubilised in a way. According to literature, it seems this milky appearance is normal, and the solubilised cellulose is described as a suspension (Ling et al., 2019; Sirviö et al., 2016), meaning it is not a true solution. Also, the ChCl-OAD solvent in the literature was observed to efficiently extract cellulose nano crystals specifically, rather than the whole cellulose sample, meaning these nanocrystals might have been solubilised here, constituting some of the 4.6% dissolved cellulose. Based on this, there might have been some solubilising effect on the cellulose. This also indicates that separating out the precipitate and calculating the yield is not the most optimal way to find the solubilisation efficacy as it does not distinguish between the different solubilising mechanisms.

A solution to this problem could be to adjust the centrifugation to e.g., 500 rpm (42 rcf) rather than 5500 rpm (5073 rcf). This could make it possible to distinguish the samples by how much precipitation

would form for each of the solvents, and how much would stay in suspension. Performing centrifugation at different speeds can also be utilised to create multiple fractionations of cellulose at different degrees of solubilisation.

Either way, none of these results indicate much of how these solvents would act on the macro fibre structure of collagen and would probably need more thorough testing to get concrete answers. It does however not indicate to have a significant effect on non-water soluble macrofibres.

4.2.3 pH of Solvents

pH measurements were performed to explore the theory that the acidity of the NADESs affects the solubilisation efficacy. Table 4 shows the resulting measurements using a standard pH meter. Double distilled water had a stabilised pH at 7.23, while CA-X-W was stable for a while at 1.74, but the value could be lower. ChCl-OAD, however, dropped below 0 and was therefore outside of the range of the instrument.

Table 4: pH value of solvents measured using a pH meter

Solvent	pH
ChCl-OAD	< 0.01
CA-X-W	1.74
Double distilled water	7.23

It is difficult to determine if these results should be trusted or not, since pH measurements are done on aqueous solutions. Each NADES are a structure of components interacting through hydrogen bonding, supposedly creating a network, and should therefore not act like a normal solution. Thus, any water present in the solvent formulations is bound in the eutectic network as well as HBDs. There are some who has explored the possibility of measuring the pH of eutectic solvents before. Jablonský & Jančíková (2023), state that the pH values of different eutectic solvents are incomparable, and it is only possible to compare pH values between solvents with the same composition, but has e.g., varying molar ratios. They elaborate that the pH of NADESs does not correspond to the concentration of hydrogen ions like in aqueous solutions, but rather the activity of hydrogen ions in the solution. The activity of hydrogen ions referring to their reactive activity. The degree of ion activity will vary significantly for each eutectic solvent, despite if the number of ions is the same in both, thus making the pH incomparable. Since the pH values should not be trusted, they suggest a different way of quantifying the acidity. Namely by the determining the acidity in milliequivalents of acid per kilogram of solvent, which is done by titration against an alkaline solution.

Still, a solvent such as CA-X-W contains around 34% water, which makes it difficult to say certainty if it is all structural water, or if the NADES is to some degree an aqueous solution. Therefore, it is of interest to explore and find out at which water concentration it stops being a structural component in the NADES, but rather a diluent. Hammond et al (2017) assessed the water in choline chloride : urea : water – based DES and found that at a 42% water concentration, the DES nanostructure was still retained to a high level, with water actively contributing to the structure. As more water was added though, the eutectic interactions would weaken non-linearly, and the nanostructure would cease completely at a 51% water content. It had transitioned to a "DES in water" system, where it became an aqueous solution. The reason this happens at a sufficiently elevated water concentrations is because it becomes preferable for the water to occupy all the hydrogen bonds around each component, thus diminishing all choline chloride – urea interaction. There is no set water concentration this happens though, and it would depend on each solvent's composition.

Similarly, Gabriele et al (2019) found that the supramolecular structure of DES was preserved until a 40-50% water concentration where it transitioned to a solution. To further support the concept that the water is bound and therefore not reactive, Abbott et al (2014) applied eutectic solvents to metals, where corrosion was not observed. Therefore, considering the water content of CA-X-W is only 34%, it can be concluded that the water present is most likely structural water and not a solution. Meaning, the pH measurements that were done using a pH meter cannot be completely trusted.

Despite the limitations of the pH measurements and the possible inaccuracies in pH values, there are reports in the literature that relied on the measurements. Wikene et al (2017) used a standard pH meter for undiluted and diluted NADESs. They did as Jablonský & Jančíková (2023) suggested and compared only pH values for the same solvent composition, but at different dilutions.

Regardless of the limitations of using a pH meter, there are some ways to get an indication of the pH values of acid – containing NADESs. Radošević et al (2018) found that NADESs containing oxalic acid are known to be the most acidic, while those with citric acid and malic acid create the second and third most acidic solvents. In terms of alkalinity, it is only those with urea that are reported to have a basic identity so far. These finding corresponds well to the measurements were ChCl-OAD has a negative acidity, and CA-X-W is less acidic, but still has a considerable acidity compared to water.

All in all, the specific pH values cannot be completely trusted as they are not aqueous solutions. If the true pH is to be found it is recommended to perform an alkaline titration to be completely sure of the results. Regardless, there is a strong indication that the NADESs have a lower pH than water. Meaning they could create an acidic environment where hydrogen ions dissociate, and consequently interacts with the collagen molecules during solubilisation.

4.2.4 Changes over storage time

During storage of the supernatants with gelatin, there was observed a change in physical properties compared to their corresponding solvents.

The gelatin in water supernatants transformed to a gel state after storage for a few days at room temperature (21°C). It was therefore necessary to remelt the samples by heating at 40°C to perform assays. This might have affected the gelatin proteins more than expected at the time. Studies show that heating a gelatinous solution to high temperatures, or moderate temperature for long periods, will lead to degradation of the collagen molecules. Resulting in smaller peptides in the solution than the original gelatin composition had. Such a degradation can already happen at 40°C (van den Bosch & Gielens, 2003). This was confirmed through physical observation when one of the supernatants had lost its ability to gel after two rounds of remelting, and the second supernatant after four rounds of reheating. By the end of all planned assays, there was only one replication left of water supernatant which would still gel, which in turn would give the most accurate results having still retained its original characteristics to some degree.

In this regard, the NADESs have an advantage as they did not gel over time, even with CA-X-W having dissolved around 99% of the gelatin, and ChCl-OAD having dissolved around 66% of the gelatin. Thus, showing a considerable higher gelatin content than the water supernatants which only had 33% of the sample dissolved. They did however have a more viscous character than the solvents themselves, and they would stay stable throughout storage with no precipitate forming. This raises the question as to

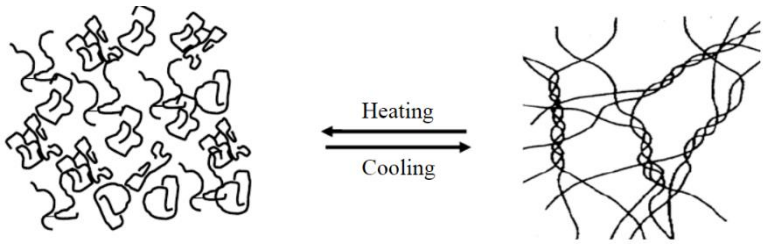


Figure 9: The gelation process visualised as dissolved collagen peptides in a heated solution partially reform the triple helices upon cooling (From: (Haug & Draget, 2009))

why they did not gel, seeing as CA-X-W contains 34% water and ChCl-OAD contains 13% water. As already explored, the water molecules in the NADES are part of a structured network of hydrogen bonds, but that still does not completely explain why no gel is forming. Gelatin in a heated solution will break the triple helixes, creating a dissolution. This breakage will be gradual until it reaches a temperature where all the helixes are broken. This is the transition temperature from helix to random coil. As the temperature sinks afterwards, there will be a partial reformation of the triple helix because

of inter-chain associations as visualised in Figure 9. These are also known as junction zones. As a result, a three-dimensional structure of crosslinks and random coils is formed. Water is present in this process by interacting with polar and charges groups as structural water. The water is then subsequently trapped in the network, now being a hydrogel (Djabourov et al., 1988; Haug & Draget, 2009). As established, water in NADESs is bound to a network, limiting it from creating a gel with gelatin. This might not be the only reason for the lack of gelation though. Grønlien et al (2020) stated that the dissolved collagen is tightly bound in the hydrogen bond network of the NADES, which would mean a high degree of solute - solvent interaction. Similarly, Cui et al (2021), looked at gelation properties of gelatin dissolved in DES, and they stated that the eutectic solvent forms extensive hydrogen bonding with the gelatin peptides, thus restricting the reformation of their helical structure upon cooling. Therefore, the lack of gelation in the supernatants could be explained by the high solute – solvent interaction not allowing any solute – solute interactions (junction zones), as well as the water being unavailable.

In section 4.4.2, the protein content of the CA-X-W supernatants were shown to be 6.5 mg/mL while the protein content of the ChCl-OAD supernatants were too low comparably. It is discussed in the same section that this is most likely due to the peptide size in the supernatant being smaller than the lower limit of detection for the assay, suggesting that proteolysis of the sample occurred. This could indicate that one of the reasons the ChCl-OAD gelatin supernatant did not gel might also have been due to the peptide size being too small to still have gelation capabilities.

4.3 Refractive index

Refractive index (RI) is a measure of how quickly light passes through a medium in relation to a reference, which usually is the speed of light in a vacuum or air (Silla et al., 2001). The RI will increase with the concentration of dissolved material in a solution, and pure water has a constant RI of 1.333 (Hosoda et al., 2004). Since NADESs are composed of components interacting as a network of hydrogen bonding, the RI measurements could indicate if they still have some characteristics of true solutions or not.

As seen in Figure 10, there seems to be some difference between the solvents but comparing the same solvent with and without dissolved sample, it is difficult to see any difference. All the values in detail can be found in Appendix 4 with standard deviations. All datapoints in Figure 10 were shown in an analysis of variance to be statistically different from each other.

Looking at the solvents themselves first, the RI increase between pure water and the NADES is not considerably large. It would therefore be interesting to investigate if there is any correlation between the RI increase and the increase in dissolved compounds between pure water and NADES. Pure water had an RI of 1.333, while CA-X-W, containing 65% dry matter had an RI of 1.440. This means for CA-X-W there was only an 8.03% increase in refractive index from pure water, even though the dry matter is 65% higher. At the same time, ChCl-OAD had a 9.23% increase in RI from water, even though the dry matter increase was as high as 86%. Used as a point of comparison, results from RI measurements

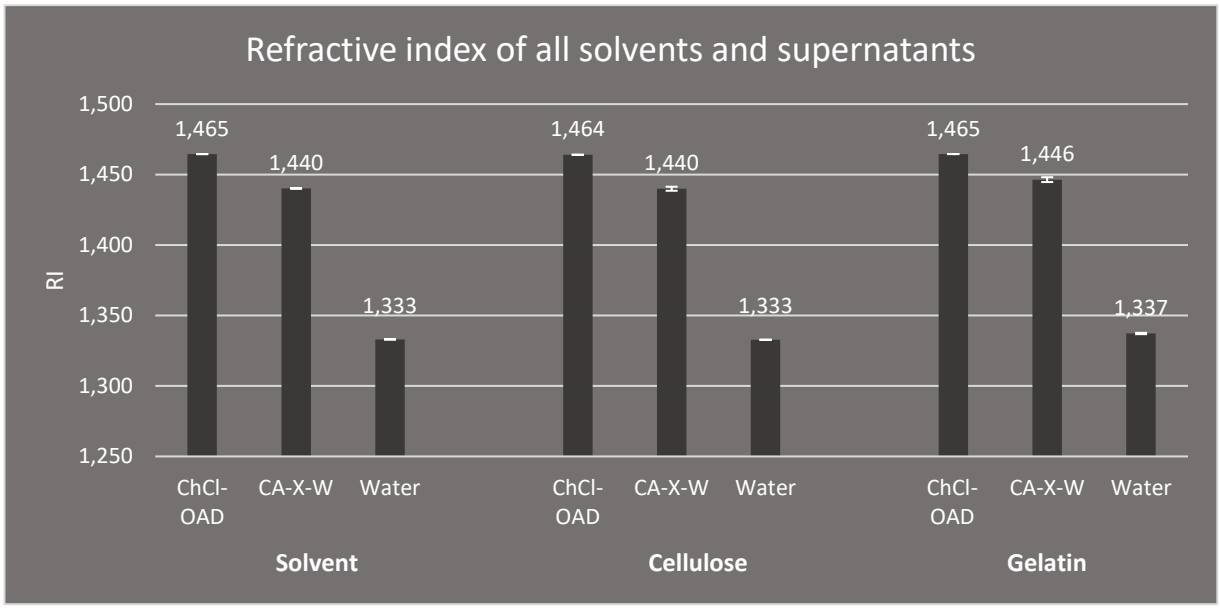


Figure 10: The average refractive index values are shown. The three columns to the left are just the solvents choline chloride : oxalic acid dihydrate (ChCl-OAD), citric acid : xylitol : water (CA-X-W) and water, the middle three are their supernatants with dissolved cellulose, and the three to the right are their supernatants with dissolved gelatin

done by Miluski & Dorosz (2006) on glucose solutions at different concentrations were used. There, a glucose solution of 1% has an RI of 1.33, while a 65% solution has an RI close to 1.45, which correlates greatly with CA-X-W reading at 1.44 for the same dry matter concentration. Based on this, it could indicate that the NADESs, at least in term of RI, acts similar to true solutions when refracting light.

The next question would then to see if the dissolved gelatin also acts like a true solution, or if they are undetected. CA-X-W read at 1.440, while with gelatin it reads at 1.446. This is a very slight RI increase, and it is difficult to conclude if it is due to the additional dry matter from gelatin. Since CA-X-W solubilised almost all the gelatin, the RI for the gelatin supernatant can be estimated. Full calculations can be found in Appendix 5. The original mixture of 18.6 grams CA-X-W and 1.4 grams gelatin before solubilisation contained 67.45% dry matter, and since it all dissolved, the assumption is that the supernatant has that same dry matter content, which is 2.45% more than the dry matter of just CA-X-W. Since there is an 8.03% increase in RI from pure water to the RI at 65% dry matter concentration, that means an 0.124% increase in RI for each 1% increase in dry matter concentration. Thus, a 2.45%

dry matter increase would mean 0.304% increase from the RI of CA-X-W to the RI of the CA-X-W gelatin supernatant. This results in a theoretical RI for solubilised gelatin in CA-X-W to be 1.444. The actual reading for gelatin was at 1.446 ± 0.001, meaning the measurement correlates quite closely with the calculated estimation. Based on this, it could mean that the solubilised gelatin also acts like a solution in terms of RI. However, these are very tiny numbers, and there might be other factors which influenced the increase in RI for dissolved gelatin compared to just the solvent.

Considering that the RI values for the NADESs correspond well to their concentration of dry matter, the solvents appear to act like solutions when refracting light. If the measurements for the CA-X-W gelatin supernatants are correct, it would indicate that the gelatin proteins when dissolved become part of the NADES structure though hydrogen bond interactions, as stated by (Grønlien et al., 2020), and thus gets measures as part of the solution. This must, however, be more closely studied to be stated confidently. Additionally, the calculations could only be done for CA-X-W, so the same conclusion cannot be generalised to the ChCl-OAD solvent or water as well.

4.4 CHARACTERISTICS OF SUPERNATANTS WITH SOLUBILISED GELATIN

All the following results are from assays exclusively performed on the supernatants of solvents which solubilised gelatin. Here the degree of proteolysis, protein content, and peptide sizes are explored.

4.4.1 Degree of proteolysis

An o-pthalaldehyde (OPA) assay was performed to give an indication of the dissolution and the degree of proteolysis on the gelatin sample. This method works in principle by quantifying the concentration of primary amino groups in each protein, peptide, and free amino acid residue in a sample (Nielsen et al., 2001). A given peptide will have a certain amount of primary amino groups, some of which are in the sidechains, and one as the N-terminus. Upon proteolysis, the concentration of primary amino groups remains the same in the sidechains but will, however, increase proportionally with the increase of formed N-terminuses. Thus, this assay could give an indication of the degree of proteolysis when comparing the same sample before and after a potentially proteolytic treatment. However, it can also be an indication of the amount of dissolution of a sample, e.g., gelatin. When dissolved, gelatin can either be all random coil, as in no intermolecular associations, or with some degree of triple helices. A high degree of random coil conformation would mean a higher degree of dissolution, which results in a higher concentration of available primary amino groups. During the assay, primary amino groups react with o-pthalaldehyde in the presence of beta-mercaptoethanol, causing a colour change

proportional to the concentration, making this a colorimetric method (Montgomery et al., 2021). Glycine was chosen for the standard curve for convenience because each molecule only has one primary amino group, making the concentrations 1:1.

When this assay was performed, the gelatin supernatants were not originally adjusted to have the same protein content. Therefore, when interpreting the results, it was not possible to determine if a high primary amino group concentration was due to there being a high degree of proteolysis or dissolution, or if it was because the protein content was high. A protein quantification assay was performed later, results presented in section 4.4.2, and these values were utilised during the data processing to normalise the primary amino group concentrations to the same protein content. The resulting graphic presentation of the primary amino group content can be seen to the right as Figure 11. All values and standard deviation can be found in Appendix 6. An analysis of variance concluded that all the supernatants have significantly different concentration of primary amino groups.

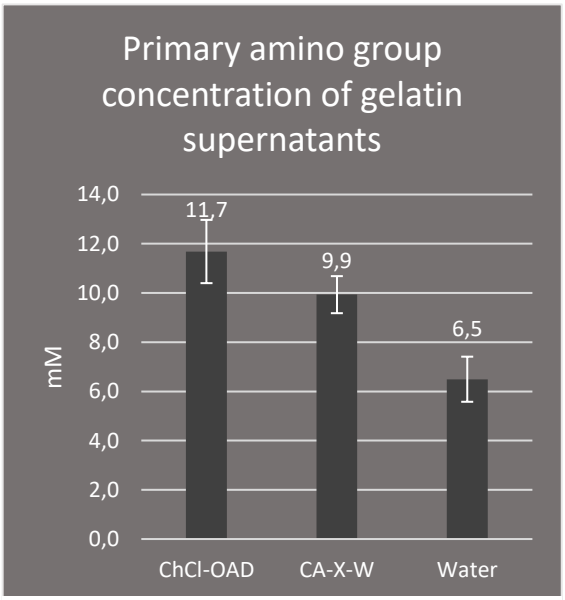


Figure 11: The primary amino group concentration in mM for the supernatants with solubilised gelatin. The results are normalised to the same protein content. To the left is supernatant of choline chloride : oxalic acid dihydrate (ChCl-OAD) containing 11.7 mM, in the middle is supernatant of citric acid : xylitol : water (CA-X-W) containing 9.9 mM, and to the right is supernatant of water containing 6.5 mM primary amino groups.

The average concentration of primary amino groups for the water supernatant is expectedly the lowest at 6.5 mM but should not be trusted as completely accurate. The low concentration of primary amino groups could indicate that this is the closest to the concentration of the original gelatin sample. However, the gelatin supernatants were, as mentioned, remelted before performing assays, which could have caused damage and degradation to the collagen molecules. As a result, the primary amino group concentration would have increased, subsequently making this value too high compared to the real concentration of the sample. For the graphic presentation in Figure 11, the first water supernatant was removed since it had stopped being able to gel. Proteolysis was confirmed by a heightened concentration of primary amino groups. These values can be found in Appendix 6. Removing this replication from the graphic presentation provides a more accurate representation of how the gelatin supernatant was from the beginning. Regardless, even if the other two supernatants still could from gels, there would be some degradation from heating, though not at excessive. Another factor which may have caused an increase in the concentration is the supernatant being subjected to heat during

the remelting right before the assay. Gelatin in heated solutions will have a higher degree of dissolution, and since the remelting was done at 40°C, it will have a higher degree of dissolution than the gelatin sample which was solubilised at 25°C. Therefore, the results presented cannot be used as a good representative of the gelatin in the water supernatants, though they might provide some indication of having less proteolysis or dissolution than the other two solvents

The results for CA-X-W were higher than those of water with an average primary amino group concentration at 9.9 mM. It would be expected for the value to be closer to that of water at 6.5 mM since this solvent has not been observed to be proteolytic. Assays performed by Batista et al (2022) on the extracted collagen would indicate that the solvent does not damage any of the functional groups in the collagen triple helix. They also assessed circular dichroism, which indicated that a partial protein denaturation might have happened during extraction. They suggest it might be due to dilute citric acid, but that was not confirmed. Similarly, Grønlien et al (2020) also observed that CA-X-W caused an unfolding of the triple helix, even at room temperature. Neither of these findings, however, suggest that there was any proteolysis of the collagen molecules occurring. Therefore, it is unlikely that proteolysis caused the high concentration of primary amino groups. The higher concentration of primary amino groups could instead be due to there being a higher degree of dissolution of the gelatin sample in CA-X-W compared to water. Which would indicate that CA-X-W can disrupt more intermolecular associations in the collagen molecules, as described by the literature, and thus possibly explaining the high solubilisation efficacy.

The ChCl-OAD supernatant contained the highest average concentration of primary amino groups at 11.7 mM. This could indicate that there has been a high degree of dissolution, which makes ChCl-OAD a solvent with similar properties as CA-X-W, if not better. However, the literature has observed that this solvent might have proteolytic capabilities instead. Bai et al (2017) described obtaining small collagen peptides because of proteolytic activity from the solvent. They used choline chloride : oxalic acid, but as explained earlier, there is reason to believe they were using oxalic acid dihydrate. Following their observation of proteolysis, they proposed an explanation as to how this solvent could have broken the peptide bonds. Stating that, hydrogen ions would react with the imino of collagen, forming a positively charged ammonium salt. This charge would attract chlorine ions by electrostatic interaction, as well as oxalic acid by hydrogen bonding. These new interactions on to the peptide would damage the inter and intra-molecular bonding of the collagen, thus causing the peptide bonds to break. Seeing as the primary amino group content for ChCl-OAD is the highest of all the solvents, it could be an indication of this same proteolytic phenomena having occurred. If that is the case, it would also support the assumption that Bai et al (2017) were using oxalic acid dihydrate.

Based on the results, there is an indication that the NADESs might cause dissolution at a higher degree than water, but they could also be proteolytic. This assay alone cannot tell which of the properties cause the heightened concentrations of primary amino groups. Additionally, there were many sources of error for this assay, which means the exact values should not be trusted fully. Instead, the OPA assay should be redone with extra care and measures to ensure more representative results. The first recommended adjustment is to perform this assay on the water supernatants very soon after extraction so that remelting only occurs once or less. The second recommendation is to adjust the samples to the same protein content ahead of performing the assay, rather than normalising in data processing like was done here. As will be explained in the next section, the protein content might also be incorrect, meaning another assay for protein content should be performed as well before redoing this assay. Other sources of error include the time sensitivity of the assay, so even if each sample was read at exactly 2 minutes after adding the OPA reagent, there could still be some inconsistencies, as well as possible protein contaminations, such as from the pipette tip.

4.4.2 Protein content

The protein content assay was done by the Bradford protein-dye binding method using the protocol developed by Bio-Rad. The results are presented graphically in Figure 12, the detailed values can be found in Appendix 7. This assay was performed originally with the purpose of adjusting the protein content of the supernatants to the same concentrations for SDS-PAGE. It can, however, also be used to see correlation with solubilisation efficacy. The latter assumes that the protein content corresponds to the amount of gelatin dissolved in each solvent. An analysis of variance concluded the protein content in the supernatants are significantly different from each other.

The supernatant of CA-X-W had an average protein content of 6.5 mg/mL and considering no proteolysis should have occurred, this would be the most accurate representation of protein content out the three solvents. Because it is the most accurate value, it is used as a point of comparison for protein content in correlation to solubilisation efficacy.

The protein content of the ChCl-OAD supernatants were on average 0.8 mg/mL, which compared to the solubilisation efficacy is too low. Considering ChCl-OAD solubilised around 66% of the gelatin, and CA-X-W dissolved 99%, the protein content should be two thirds the protein content of CA-X-W, hence around 4.3 mg/mL. There is a possible explanation for this. The protein content assay was chosen because it is very quick to perform, and the results were not intended to be presented. Problem is, the Bradford method has a weakness where the lower limit of detection is 3-5 kDa (Nobel, 2000). Thus, causing all small peptides to be undetected. If this is true, it would mean that the hypothesis of

proteolysis might be true, seeing as there is no other explanation as to why the protein content would be so low. However, if the protein content of ChCl-OAD is supposed to be larger, then the normalisation performed in the previous section would be inaccurate as well. Assuming the protein content should be higher, then the primary amino group concentration, and consequently the degree of proteolysis, would be normalised lower, perhaps even lower than the supernatant of CA-X-W. Which might contradict the theory of proteolysis being a prominent characteristic of this solvent. Still, the primary amino group content assay most likely had some sources of error making the results quite unreliable until they are redone.

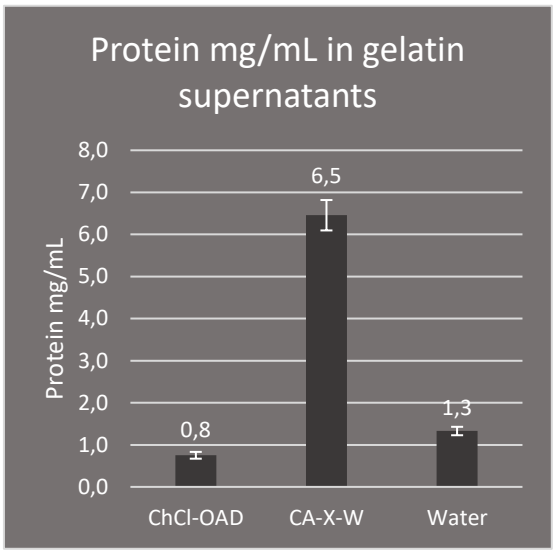


Figure 12: The protein content of supernatants with dissolved gelatin given as mg/mL supernatant. The choline chloride : oxalic acid dihydrate (ChCl-OAD) supernatant contained 0.8 mg/mL protein, while citric acid : xylitol : water (CA-X-W) had 6.5 mg/mL, and the water supernatant contained 1.3 mg/mL protein

The average protein content of the water supernatants was also quite low at 1.3 mg/mL. According to the solubilisation efficacy of water and CA-X-W, the protein content in the water supernatant should be a third of the protein content in CA-X-W, meaning it should instead be around 2.1 mg/mL. As mentioned, the water supernatants were remelted, which likely led to proteolysis. Like with ChCl-OAD, this might have created very small peptides, which were not detected by the protein assay. Thus, making the protein content lower than it should be. Here, as well, the first supernatant of water was removed as gelation properties were lost due to extensive proteolysis. Looking at the complete results in Appendix 7, the protein content for this supernatant replication is even lower at round 0.3 mg/mL. Which further supports the theory of extensive proteolysis having occurred due to multiple rounds of remelting. These low values also explain why the first water supernatant for primary amino groups in Appendix 6 is so high as the normalisation would be inaccurate with this protein content.

Going forward, it would be smart to consider a different protein assay to obtain more accurate results than those presented. One assay that can be used instead is the Bicinchoninic Acid Assay (BCA) method. For this assay there is no reported lower limit for peptide size, but the lower limit for detecting protein amount is at 20 µg/mL (Scientific, 2013). Another assay to consider is performing the Kjeldahl method, which quantifies the amount nitrogen present in the sample, from which the protein content can be calculated (Jiang et al., 2014).

4.4.3 Peptide characteristics of supernatants

There was indication that there might have been some proteolytic activity among the solvents from the OPA assay in section 4.4.1. Because it was not clear if the primary amino groups detected were due to proteolysis or extensive dissolution, SDS-PAGE was run to investigate it further. By comparing the sizes of the peptides in the supernatants it would be possible to see if the size ranges were similar between the solvents, meaning no proteolysis, or if a solvent caused smaller peptides, indicating proteolytic activity. The resulting gel from the electrophoresis is shown in Figure 13. The wells that are shown were selected for having the clearest bands.

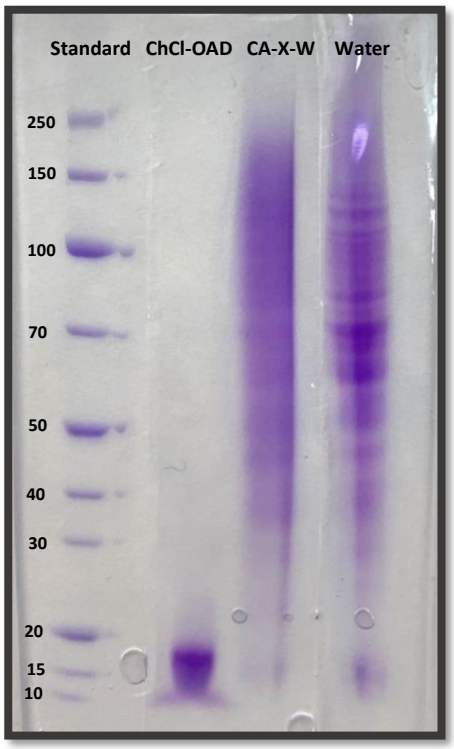


Figure 13: Picture of gel showing the protein standard to the left, then the choline chloride : oxalic acid dihydrate (ChCl-OAD) gelatin supernatant, followed by the citric acid : xylitol : water (CA-X-W) gelatin supernatant, and lastly to the right, the supernatant of gelatin in water. The bands of the standard are marked with the kDa value they represent.

The middle well contains the supernatant of CA-X-W, which notably has a broad range of peptide sizes. These results confirm that the solvent is not proteolytic and the heightened concentration in primary amino groups observed in section 4.4.1 is likely caused by a high degree of dissolution. Considering CA-X-W dissolved all the gelatin sample, and is non-destructive, this will be used as a point of reference for the other two solvents as it might be the most representative. Furthermore, the sample used was gelatin, not collagen, meaning there will not be very clear bands representing the key collagen molecule constituents due to the hydrolysis. However, from the works of Batista et al (2022), there is an SDS-PAGE gel showing two bands between 100 kDa and 150 kDa representing α1 and α2 collagen strands. Though it is not easy to see on this gel, there are two very faint bands in the same area they reported theirs. This assay was performed twice, and in the other gel, found in Appendix 8, these two bands were much more prominent. As this is a qualitative assay, it is not possible to determine if these bands are the same as those in Batista et al (2022) representing the α1 and α2 collagen strands. However, it gives a small indication that some of the collagen characteristics might be maintained in the gelatin sample.

The first well, excluding the standard, contains the gelatin supernatant of ChCl-OAD. Here, there are no clear bands, and it appears that almost all the peptides are smaller than 20 kDa. By looking at the peptide sizes of CA-X-W, most sizes of the original gelatin sample should be a lot larger, with the majority being between 50 kDa and 150 kDa. The fact that all the peptides for ChCl-OAD are so small

further supports the idea that the solvent has had a proteolytic effect on the protein. The small peptide sizes cannot be explained by the ChCl-OAD solvent selectively only dissolving small peptides, because then these low bands should be visible for CA-X-W as well, having dissolved 99% of the sample. Therefore, the most likely explanation is that the peptides were proteolysed, which are the same observations as Bai et al (2017). They also performed SDS-PAGE and observed most peptides from this solvent to be smaller than 11 kDa, which corresponds well with the findings here. Furthermore, this could also support the theory that the solvent they used was not ChCl-OA (oxalic acid), but in fact ChCl-OAD.

The last well shows the water supernatants, which, like CA-X-W, has a wide range of peptide sizes. Therefore, the collagen molecules here were also quite preserved. However, due to the supernatant being remelted there was some proteolysis occurring, which can be observed by the collection of bands found at below 20 kDa. As mentioned, around 33% of the gelatin was solubilised at 25°C after 4 hours of shaking. It was explained that the dissolution of gelatin in water happens at a temperature range, which is why some solubilised even at 25°C. It was however not indicated anything about what the characteristics of those peptides were, nor was it clear if the solubilised peptide sizes were representative of the whole gelatin sample. Since the peptide size distribution is very similar to that of CA-X-W, where almost all the gelatin dissolved, it would indicate that the gelatin peptides solubilised in the water supernatant was quite evenly distributed, and not of a few size ranges.

Overall, the gel electrophoresis confirms most of the earlier findings. The CA-X-W solvent preserved the gelatin structure while also providing a high degree of dissolution. The water supernatants similarly had a good distribution of peptide sizes, but also some small ones due to degradation by heat. Lastly, the gel confirmed that ChCl-OAD caused proteolysis of the gelatin proteins as observed by all the peptides being less than 20 kDa.

5 CONCLUSION

The aim of this project was to find potential NADES formulations which can be applied for the extraction of collagen, and to assess their physical and solubilising properties by using gelatin and cellulose as representative sample materials. Two formulations were found which could solubilise gelatin samples better than water, ChCl-OAD and CA-X-W. They were both notably quick and easy to synthesise and were characterised by low viscosities, making them energy efficient and suitable for extraction processes. The CA-X-W solvent, which had the highest efficacy, solubilised 99.5% of the gelatin samples. The pH of the solvent was measured to be low, which according to the literature, may contribute to the solubilising effect. CA-X-W was found to cause a high degree of dissolution of the gelatin sample, because of the high concentration of primary amino groups. Literature states this is due to a high degree of solute – solvent interactions. This trait was expressed in the lack of gelation during storage, and through the dissolved gelatin acting as part of the hydrogen bonding in the eutectic network for the refractive index measurements. Lastly, this solvent was stated in the literature to be non-destructive. This was confirmed by the wide peptide size range in the SDS-PAGE assay. Thus, the extracted collagen should still have its desired properties making this the most promising solvent for industrial application. The other solvent ChCl-OAD dissolved 66.1% of the gelatin sample and is also known to solubilise collagen from the literature. Proteolysis appears to be the main factor for the dissolution and is theorised to have occurred due to hydrogen ions from the solvent destabilising the peptide bonds. During storage, the proteolysed peptides in the supernatant could no longer gel, and the SDS-PAGE assay showed only peptide sizes below 20 kDa. Additionally, there was a high concentration of primary amino groups, and the protein content was very low, due to the peptide size being below the lower detection limit. Due to the small peptide size, the desired gelation properties are most likely lost, which could make the extraction product undesirable. However, they could be researched as potential bioactive compounds in the biomedical field. Neither of the solvents had a significant solubilisation of cellulose compared to water, which could indicate that they would target the collagen macrofibre structure during solubilisation.

Overall, both solvents show very promising characteristics for the solubilisation of collagen and are great candidates as an alternative to the conventional acid-based extraction method.

6 FUTURE WORK AND PERSPECTIVES

Continuing this project, it would be necessary to gain more information about these solvents. Firstly, the solvent should be used for the extraction of collagen from a biological matrix. This will better indicate the true solubilisation efficacies of collagen in its native form, making it easier to evaluate if these solvents will provide a preferable alternative for today's method. The extracted collagens should be further assessed for e.g., their properties and pureness as it would provide a better understanding of how the extraction product can be utilised. Secondly, it will be crucial to study the toxicity of the NADESs as this could be a potential limitation for the extraction product to be approved for human consumption.

7 REFERENCES

Abbott, A. P., Ahmed, E. I., Harris, R. C., & Ryder, K. S. (2014). Evaluating water miscible deep eutectic solvents (DESs) and ionic liquids as potential lubricants. *Green Chem.*, *16*(9), 4156–4161. https://doi.org/10.1039/C4GC00952E

Abbott, A. P., Capper, G., Davies, D. L., Rasheed, R. K., & Tambyrajah, V. (2003). Novel solvent properties of choline chloride/urea mixturesElectronic supplementary information (ESI) available: spectroscopic data. See http://www.rsc.org/suppdata/cc/b2/b210714g/. *Chemical Communications*, *1*, 70–71. https://doi.org/10.1039/b210714g

Bai, C., Wei, Q., & Ren, X. (2017). Selective Extraction of Collagen Peptides with High Purity from Cod Skins by Deep Eutectic Solvents. *ACS Sustainable Chemistry & Engineering*, *5*(8), 7220–7227. https://doi.org/10.1021/acssuschemeng.7b01439

Batista, M. P., Fernández, N., Gaspar, F. B., Bronze, M. do R., & Duarte, A. R. C. (2022). Extraction of Biocompatible Collagen From Blue Shark Skins Through the Conventional Extraction Process Intensification Using Natural Deep Eutectic Solvents. *Frontiers in Chemistry*, *10*. https://doi.org/10.3389/fchem.2022.937036

Benjakul, S., Oungbho, K., Visessanguan, W., Thiansilakul, Y., & Roytrakul, S. (2009). Characteristics of gelatin from the skins of bigeye snapper, Priacanthus tayenus and Priacanthus macracanthus. *Food Chemistry*, *116*(2), 445–451. https://doi.org/10.1016/j.foodchem.2009.02.063

Benvenutti, L., Zielinski, A. A. F., & Ferreira, S. R. S. (2019). Which is the best food emerging solvent: IL, DES or NADES? *Trends in Food Science & Technology*, *90*, 133–146. https://doi.org/10.1016/j.tifs.2019.06.003

Bisht, M., Martins, M., Dias, A. C. R. V., Ventura, S. P. M., & Coutinho, J. A. P. (2021). Uncovering the potential of aqueous solutions of deep eutectic solvents on the extraction and purification of collagen type I from Atlantic codfish (*Gadus morhua*). *Green Chemistry*, *23*(22), 8940–8948. https://doi.org/10.1039/D1GC01432C

Campiglio, C. E., Contessi Negrini, N., Farè, S., & Draghi, L. (2019). Cross-Linking Strategies for Electrospun Gelatin Scaffolds. *Materials*, *12*(15), 2476. https://doi.org/10.3390/ma12152476

Choi, Y. H., van Spronsen, J., Dai, Y., Verberne, M., Hollmann, F., Arends, I. W. C. E., Witkamp, G.-J., & Verpoorte, R. (2011). Are Natural Deep Eutectic Solvents the Missing Link in Understanding Cellular Metabolism and Physiology? *Plant Physiology*, *156*(4), 1701–1705. https://doi.org/10.1104/pp.111.178426

Choline - Fact Sheet for Health Professionals. (2022). National Institutes of Health. https://ods.od.nih.gov/factsheets/Choline-HealthProfessional/

Coppola, D., Oliviero, M., Vitale, G. A., Lauritano, C., D'Ambra, I., Iannace, S., & de Pascale, D. (2020). Marine Collagen from Alternative and Sustainable Sources: Extraction, Processing and Applications. *Marine Drugs*, *18*(4), 214. https://doi.org/10.3390/md18040214

Craveiro, R., Aroso, I., Flammia, V., Carvalho, T., Viciosa, M. T., Dionísio, M., Barreiros, S., Reis, R. L., Duarte, A. R. C., & Paiva, A. (2016). Properties and thermal behavior of natural deep eutectic

solvents. *Journal of Molecular Liquids*, *215*, 534–540. https://doi.org/10.1016/j.molliq.2016.01.038

Cui, Y., Zhu, Y., Dai, R., Shan, Z., Yi, J., & Chen, H. (2021). The solubility and interactions of gelatin in "water-in-sodium acetate trihydrate/urea-DES" system. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *625*, 126916. https://doi.org/10.1016/j.colsurfa.2021.126916

Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., & Choi, Y. H. (2013). Natural deep eutectic solvents as new potential media for green technology. *Analytica Chimica Acta*, *766*, 61–68. https://doi.org/10.1016/j.aca.2012.12.019

Dai, Y., Varypataki, E. M., Golovina, E. A., Jiskoot, W., Witkamp, G.-J., Choi, Y. H., & Verpoorte, R. (2021). *Natural deep eutectic solvents in plants and plant cells: In vitro evidence for their possible functions* (pp. 159–184). https://doi.org/10.1016/bs.abr.2020.09.012

Deshmukh, K., Basheer Ahamed, M., Deshmukh, R. R., Khadheer Pasha, S. K., Bhagat, P. R., & Chidambaram, K. (2017). Biopolymer Composites With High Dielectric Performance: Interface Engineering. In *Biopolymer Composites in Electronics* (pp. 27–128). Elsevier. https://doi.org/10.1016/B978-0-12-809261-3.00003-6

Dickerson, A. S., Lee, J. S., Keshava, C., Hotchkiss, A., & Persad, A. S. (2018). Assessment of Health Effects of Exogenous Urea: Summary and Key Findings. *Current Environmental Health Reports*, *5*(2), 205–212. https://doi.org/10.1007/s40572-018-0198-8

Djabourov, M., Leblond, J., & Papon, P. (1988). Gelation of aqueous gelatin solutions. I. Structural investigation. *Journal de Physique*, *49*(2), 319–332. https://doi.org/10.1051/jphys:01988004902031900

Douard, L., Bras, J., Encinas, T., & Belgacem, M. N. (2021). Natural acidic deep eutectic solvent to obtain cellulose nanocrystals using the design of experience approach. *Carbohydrate Polymers*, *252*, 117136. https://doi.org/10.1016/j.carbpol.2020.117136

Du, C., Zhao, B., Chen, X.-B., Birbilis, N., & Yang, H. (2016). Effect of water presence on choline chloride-2urea ionic liquid and coating platings from the hydrated ionic liquid. *Scientific Reports*, *6*(1), 29225. https://doi.org/10.1038/srep29225

El Achkar, T., Greige-Gerges, H., & Fourmentin, S. (2021). Basics and properties of deep eutectic solvents: a review. *Environmental Chemistry Letters*, *19*(4), 3397–3408. https://doi.org/10.1007/s10311-021-01225-8

ER No. 1223/2009 of the European Parliament and of the Council of 30 November 2009 on Cosmetic Products, (2009).

Gabriele, F., Chiarini, M., Germani, R., Tiecco, M., & Spreti, N. (2019). Effect of water addition on choline chloride/glycol deep eutectic solvents: Characterization of their structural and physicochemical properties. *Journal of Molecular Liquids*, *291*, 111301. https://doi.org/10.1016/j.molliq.2019.111301

Gelse, K. (2003). Collagens—structure, function, and biosynthesis. *Advanced Drug Delivery Reviews*, *55*(12), 1531–1546. https://doi.org/10.1016/j.addr.2003.08.002

Gilsenan, P. (2000). Rheological characterisation of gelatins from mammalian and marine sources. *Food Hydrocolloids*, *14*(3), 191–195. https://doi.org/10.1016/S0268-005X(99)00050-8

Gnanamani, A., Shanmugavel, M., Ghosh, P., & Radhakrishnan, R. (2020). *Poultry spent wastes: an emerging trend in collagen mining*. *6*, 26–35. https://doi.org/10.15406/atroa.2020.06.00113

Gomez, F. J. V., Espino, M., Fernández, M. A., & Silva, M. F. (2018). A Greener Approach to Prepare Natural Deep Eutectic Solvents. *ChemistrySelect*, *3*(22), 6122–6125. https://doi.org/10.1002/slct.201800713

Gómez-Guillén, M. C., Giménez, B., López-Caballero, M. E., & Montero, M. P. (2011). Functional and bioactive properties of collagen and gelatin from alternative sources: A review. *Food Hydrocolloids*, *25*(8), 1813–1827. https://doi.org/10.1016/j.foodhyd.2011.02.007

Grønlien, K. G., Pedersen, M. E., & Tønnesen, H. H. (2020). A natural deep eutectic solvent (NADES) as potential excipient in collagen-based products. *International Journal of Biological Macromolecules*, *156*, 394–402. https://doi.org/10.1016/j.ijbiomac.2020.04.026

Gumrah Dumanli, A. (2017). Nanocellulose and its Composites for Biomedical Applications. *Current Medicinal Chemistry*, *24*(5), 512–528. https://doi.org/10.2174/0929867323666161014124008

Hammond, O. S., Bowron, D. T., & Edler, K. J. (2017). The Effect of Water upon Deep Eutectic Solvent Nanostructure: An Unusual Transition from Ionic Mixture to Aqueous Solution. *Angewandte Chemie International Edition*, *56*(33), 9782–9785. https://doi.org/10.1002/anie.201702486

Haug, I. J., & Draget, K. I. (2009). 6 - Gelatin. In G. O. Phillips & P. A. Williams (Eds.), *Handbook of Hydrocolloids (Second Edition)* (pp. 142–163). Woodhead Publishing. https://doi.org/https://doi.org/10.1533/9781845695873.142

Hayyan, M., Hashim, M. A., Hayyan, A., Al-Saadi, M. A., AlNashef, I. M., Mirghani, M. E. S., & Saheed, O. K. (2013). Are deep eutectic solvents benign or toxic? *Chemosphere*, *90*(7), 2193–2195. https://doi.org/10.1016/j.chemosphere.2012.11.004

Hosoda, H., Mori, H., Sogoshi, N., Nagasawa, A., & Nakabayashi, S. (2004). Refractive Indices of Water and Aqueous Electrolyte Solutions under High Magnetic Fields. *The Journal of Physical Chemistry A*, *108*(9), 1461–1464. https://doi.org/10.1021/jp0310145

Huang, W.-C., Zhao, D., Guo, N., Xue, C., & Mao, X. (2018). Green and Facile Production of Chitin from Crustacean Shells Using a Natural Deep Eutectic Solvent. *Journal of Agricultural and Food Chemistry*, *66*(45), 11897–11901. https://doi.org/10.1021/acs.jafc.8b03847

interchim. (n.d.). OPA, amine detection reagent. In *FluoProbes*. Retrieved 23 April 2023, from https://www.interchim.fr/ft/0/02727A.pdf

Jablonský, M., & Jančíková, V. (2023). Comparison of the acidity of systems based on choline chloride and lactic acid. *Journal of Molecular Liquids*, *380*, 121731. https://doi.org/10.1016/j.molliq.2023.121731

Jiang, B., Tsao, R., Li, Y., & Miao, M. (2014). Food Safety: Food Analysis Technologies/Techniques. In *Encyclopedia of Agriculture and Food Systems* (pp. 273–288). Elsevier. https://doi.org/10.1016/B978-0-444-52512-3.00052-8

Kalhor, P., & Ghandi, K. (2019). Deep Eutectic Solvents for Pretreatment, Extraction, and Catalysis of Biomass and Food Waste. *Molecules*, *24*(22), 4012. https://doi.org/10.3390/molecules24224012

Keenan, T. R. (2012). Gelatin. In *Polymer Science: A Comprehensive Reference* (pp. 237–247). Elsevier. https://doi.org/10.1016/B978-0-444-53349-4.00265-X

Kumar, A. K., Parikh, B. S., & Pravakar, M. (2016). Natural deep eutectic solvent mediated pretreatment of rice straw: bioanalytical characterization of lignin extract and enzymatic hydrolysis of pretreated biomass residue. *Environmental Science and Pollution Research*, *23*(10), 9265–9275. https://doi.org/10.1007/s11356-015-4780-4

Ling, Z., Edwards, J. V., Guo, Z., Prevost, N. T., Nam, S., Wu, Q., French, A. D., & Xu, F. (2019). Structural variations of cotton cellulose nanocrystals from deep eutectic solvent treatment: micro and nano scale. *Cellulose*, *26*(2), 861–876. https://doi.org/10.1007/s10570-018-2092-9

Liu, Y., Friesen, J. B., McAlpine, J. B., Lankin, D. C., Chen, S.-N., & Pauli, G. F. (2018). Natural Deep Eutectic Solvents: Properties, Applications, and Perspectives. *Journal of Natural Products*, *81*(3), 679–690. https://doi.org/10.1021/acs.jnatprod.7b00945

Lorenzetti, A. S., Lo Fiego, M. J., Silva, M. F., Domini, C., & Gomez, F. J. V. (2022). Water behavior study for tailoring fructose-citric acid based natural deep eutectic solvent properties towards antibiotics solubilization. *Journal of Molecular Liquids*, *363*, 119917. https://doi.org/10.1016/j.molliq.2022.119917

Matinong, A. M. E., Chisti, Y., Pickering, K. L., & Haverkamp, R. G. (2022). Collagen Extraction from Animal Skin. *Biology*, *11*(6), 905. https://doi.org/10.3390/biology11060905

McDonald, S., Wang, B., Donell, F., Ramirez, J., & Brooks, J. (n.d.). *DRY WEIGHT DETERMINATION OF TISSUES*. Retrieved 12 May 2023, from https://www.tdi-bi.com/analytical_services/environmental/NOAA_methods/Tissue%20Dry%20Weigt%20Determination.pdf

Meng, X., Ballerat-Busserolles, K., Husson, P., & Andanson, J.-M. (2016). Impact of water on the melting temperature of urea + choline chloride deep eutectic solvent. *New Journal of Chemistry*, *40*(5), 4492–4499. https://doi.org/10.1039/C5NJ02677F

Meng, Z., Zhao, J., Duan, H., Guan, Y., & Zhao, L. (2018). Green and efficient extraction of four bioactive flavonoids from Pollen Typhae by ultrasound-assisted deep eutectic solvents extraction. *Journal of Pharmaceutical and Biomedical Analysis*, *161*, 246–253. https://doi.org/10.1016/j.jpba.2018.08.048

Miluski, P., & Dorosz, D. (2006). *Measurement of refractive index using capillary waveguide* (R. S. Romaniuk, Ed.; pp. 634716-634716–6). https://doi.org/10.1117/12.714633

Mirondo, R., & Barringer, S. (2015). Improvement of Flavor and Viscosity in Hot and Cold Break Tomato Juice and Sauce by Peel Removal. *Journal of Food Science*, *80*(1), S171–S179. https://doi.org/10.1111/1750-3841.12725

Montgomery, V. A., Lindsey, C. Y., Smith, L. A., & Webb, R. P. (2021). Development of an o-pthalaldehyde (OPA) assay to measure protein content in Ricin Vaccine E. coli (RVEc™). *Vaccine*, *39*(3), 564–570. https://doi.org/10.1016/j.vaccine.2020.12.008

Muthuraj, R., Jimenez-Saelices, C., Grohens, Y., & Seantier, B. (2018). *Chapter 15. Applications of Polysaccharide and Protein Based Aerogels in Thermal Insulation* (pp. 261–294). https://doi.org/10.1039/9781782629979-00261

Naz, S., Ahmad, N., Akhtar, J., Ahmad, N. M., Ali, A., & Zia, M. (2016). Management of citrus waste by switching in the production of nanocellulose. *IET Nanobiotechnology*, *10*(6), 395–399. https://doi.org/10.1049/iet-nbt.2015.0116

Nielsen, P. M., Petersen, D., & Dambmann, C. (2001). Improved Method for Determining Food Protein Degree of Hydrolysis. *Journal of Food Science*, *66*(5), 642–646. https://doi.org/10.1111/j.1365-2621.2001.tb04614.x

Nobel, A. (2000). *Quick Start™ Bradford Protein Assay Instruction Manual*.

Paiva, A., Craveiro, R., Aroso, I., Martins, M., Reis, R. L., & Duarte, A. R. C. (2014). Natural Deep Eutectic Solvents – Solvents for the 21st Century. *ACS Sustainable Chemistry & Engineering*, *2*(5), 1063–1071. https://doi.org/10.1021/sc500096j

Pal, G. K., & Suresh, P. V. (2016). Sustainable valorisation of seafood by-products: Recovery of collagen and development of collagen-based novel functional food ingredients. *Innovative Food Science & Emerging Technologies*, *37*, 201–215. https://doi.org/10.1016/j.ifset.2016.03.015

Radošević, K., Čanak, I., Panić, M., Markov, K., Bubalo, M. C., Frece, J., Srček, V. G., & Redovniković, I. R. (2018). Antimicrobial, cytotoxic and antioxidative evaluation of natural deep eutectic solvents. *Environmental Science and Pollution Research*, *25*(14), 14188–14196. https://doi.org/10.1007/s11356-018-1669-z

Radošević, K., Cvjetko Bubalo, M., Gaurina Srček, V., Grgas, D., Landeka Dragičević, T., & Radojčić Redovniković, I. (2015). Evaluation of toxicity and biodegradability of choline chloride based deep eutectic solvents. *Ecotoxicology and Environmental Safety*, *112*, 46–53. https://doi.org/10.1016/j.ecoenv.2014.09.034

Rashid, R., Mohd Wani, S., Manzoor, S., Masoodi, F. A., & Masarat Dar, M. (2023). Green extraction of bioactive compounds from apple pomace by ultrasound assisted natural deep eutectic solvent extraction: Optimisation, comparison and bioactivity. *Food Chemistry*, *398*, 133871. https://doi.org/10.1016/j.foodchem.2022.133871

Rente, D., Paiva, A., & Duarte, A. R. (2021). The Role of Hydrogen Bond Donor on the Extraction of Phenolic Compounds from Natural Matrices Using Deep Eutectic Systems. *Molecules*, *26*(8), 2336. https://doi.org/10.3390/molecules26082336

Rodriguez, N. R., van den Bruinhorst, A., Kollau, L. J. B. M., Kroon, M. C., & Binnemans, K. (2019). Degradation of Deep-Eutectic Solvents Based on Choline Chloride and Carboxylic Acids. *ACS Sustainable Chemistry & Engineering*, *7*(13), 11521–11528. https://doi.org/10.1021/acssuschemeng.9b01378

Sadowska, M., Kołodziejska, I., & Niecikowska, C. (2003). Isolation of collagen from the skins of Baltic cod (Gadus morhua). *Food Chemistry*, *81*(2), 257–262. https://doi.org/10.1016/S0308-8146(02)00420-X

Santana, A. P. R., Mora-Vargas, J. A., Guimarães, T. G. S., Amaral, C. D. B., Oliveira, A., & Gonzalez, M. H. (2019). Sustainable synthesis of natural deep eutectic solvents (NADES) by different methods. *Journal of Molecular Liquids*, *293*, 111452. https://doi.org/10.1016/j.molliq.2019.111452

Schmidt, M. M., Dornelles, R. C. P., Mello, R. O., Kubota, E. H., Mazutti, M. A., Kempka, A. P., & Demiate, I. M. (2016). Collagen extraction process. *International Food Research Journal*, *23*(3),

913–922. https://www.proquest.com/scholarly-journals/collagen-extraction-process/docview/1789076079/se-2?accountid=12870

Scientific, T. (2013). Pierce BCA protein assay kit. *Pierce BCA*, *449*.

Shanks, R. A. (2014). Chemistry and structure of cellulosic fibres as reinforcements in natural fibre composites. In *Natural Fibre Composites* (pp. 66–83). Elsevier. https://doi.org/10.1533/9780857099228.1.66

Shoulders, M. D., & Raines, R. T. (2009). Collagen Structure and Stability. *Annual Review of Biochemistry*, *78*(1), 929–958. https://doi.org/10.1146/annurev.biochem.77.032207.120833

Silla, E., Arnau, A., & TuñóN, I. (2001). Fundamental principles governing solvents use. *Handbook of Solvents*, *7*.

Sirviö, J. A., Visanko, M., & Liimatainen, H. (2016). Acidic Deep Eutectic Solvents As Hydrolytic Media for Cellulose Nanocrystal Production. *Biomacromolecules*, *17*(9), 3025–3032. https://doi.org/10.1021/acs.biomac.6b00910

Skarpalezos, D., & Detsi, A. (2019). Deep Eutectic Solvents as Extraction Media for Valuable Flavonoids from Natural Sources. *Applied Sciences*, *9*(19), 4169. https://doi.org/10.3390/app9194169

Smith, E. L., Abbott, A. P., & Ryder, K. S. (2014). Deep Eutectic Solvents (DESs) and Their Applications. *Chemical Reviews*, *114*(21), 11060–11082. https://doi.org/10.1021/cr300162p

Telis, V., Wolf, K., & Sobral, P. (2006). Characterizations of Collagen Fibers for Biodegradable Films Production. *13th World Congress of Food Science & Technology*. https://doi.org/10.1051/IUFoST:20060929

van den Bosch, E., & Gielens, C. (2003). Gelatin degradation at elevated temperature. *International Journal of Biological Macromolecules*, *32*(3–5), 129–138. https://doi.org/10.1016/S0141-8130(03)00046-1

Van Vlierberghe, S., Graulus, G.-J., Keshari Samal, S., Van Nieuwenhove, I., & Dubruel, P. (2014). Porous hydrogel biomedical foam scaffolds for tissue repair. In *Biomedical Foams for Tissue Engineering Applications* (pp. 335–390). Elsevier. https://doi.org/10.1533/9780857097033.2.335

Wang, J. K., Yeo, K. P., Chun, Y. Y., Tan, T. T. Y., Tan, N. S., Angeli, V., & Choong, C. (2017). Fish scale-derived collagen patch promotes growth of blood and lymphatic vessels in vivo. *Acta Biomaterialia*, *63*, 246–260. https://doi.org/10.1016/j.actbio.2017.09.001

Wikene, K. O., Rukke, H. V., Bruzell, E., & Tønnesen, H. H. (2017). Investigation of the antimicrobial effect of natural deep eutectic solvents (NADES) as solvents in antimicrobial photodynamic therapy. *Journal of Photochemistry and Photobiology B: Biology*, *171*, 27–33. https://doi.org/10.1016/j.jphotobiol.2017.04.030

Yang, J., Ding, C., Tang, L., Deng, F., Yang, Q., Wu, H., Chen, L., Ni, Y., Huang, L., & Zhang, M. (2020). Novel Modification of Collagen: Realizing Desired Water Solubility and Thermostability in a Conflict-Free Way. *ACS Omega*, *5*(11), 5772–5780. https://doi.org/10.1021/acsomega.9b03846

Yao, X.-H., Zhang, D.-Y., Duan, M.-H., Cui, Q., Xu, W.-J., Luo, M., Li, C.-Y., Zu, Y.-G., & Fu, Y.-J. (2015). Preparation and determination of phenolic compounds from Pyrola incarnata Fisch. with a

green polyols based-deep eutectic solvent. *Separation and Purification Technology*, *149*, 116–123. https://doi.org/10.1016/j.seppur.2015.03.037

Zhang, Q., De Oliveira Vigier, K., Royer, S., & Jérôme, F. (2012). Deep eutectic solvents: syntheses, properties and applications. *Chemical Society Reviews*, *41*(21), 7108. https://doi.org/10.1039/c2cs35178a

Zhang, S., Zhang, T., & Tang, S. (2016). Determination of the Hammett Acidity Functions of Triflic Acid/Ionic Liquid Binary Mixtures by the ^{13} C NMR-Probe Method. *Journal of Chemical & Engineering Data*, *61*(6), 2088–2097. https://doi.org/10.1021/acs.jced.6b00015

Zhao, Z., Chen, X., Ali, M. F., Abdeltawab, A. A., Yakout, S. M., & Yu, G. (2018). Pretreatment of wheat straw using basic ethanolamine-based deep eutectic solvents for improving enzymatic hydrolysis. *Bioresource Technology*, *263*, 325–333. https://doi.org/10.1016/j.biortech.2018.05.016

Zhekenov, T., Toksanbayev, N., Kazakbayeva, Z., Shah, D., & Mjalli, F. S. (2017). Formation of type III Deep Eutectic Solvents and effect of water on their intermolecular interactions. *Fluid Phase Equilibria*, *441*, 43–48. https://doi.org/10.1016/j.fluid.2017.01.022

8 APPENDIX

Appendix 1 – OPA assay protocol

Protocol 1 : Protein and peptide assay

This protocol is designed to measure amine content in peptides and proteins to control the degree of labeling or conjugation (differential amine content before and after labeling). α-acetytl-lysine can be used as standard, containing 1 amine per molecule. A ratio of labeling can then be determined.

1- Prepare a 50mM carbonate pH10.5 buffer

2- Prepare the working reagent :
5mg of OPA + 100µl of pure Ethanol + 5µl of b-2-mercatoethanol + 10ml of 50mM carbonate buffer pH10.5

This OPA reagent should be protected from direct light, and used within 2 hours. It can eventually be stored under nitrogen in ambered glass vials for 1-2 weeks at 4°C.

3- Prepare the standards:
Prepare a 10mM acetylLysine (09111A) standard solution in water (18.8mg αacLys + 1ml dH_2O)
Prepare serial dilutions of 0.8 to 12µM of αacLys standard solution in carbonate buffer.
Rem : for accurate quantitation, a standard curve may be prepared with a purified peptide or protein

4- Prepare samples dilutions in carbonate buffer.

5- Assay:
Pipette 100µl of sample in clean disposable tubes.
Rem : plastic tubes of bad quality may produce background signal.
Check that OD of a blank is quite stable within 0+5min

6- Add 1ml of OPA reagent (1-), incubate at room temperature for 2min:

7- Place the solution in spectrophotometer, and read absorbance at 340nm at exactly 2min
Rem : readings should be performed after the same incubation duration for greater accuracy. ODs are sufficiently stable between 1min and 2minutes for αacLys and 2-4min for proteins. The incubation period may so be optimized, down 1min30 and up 10min, depending of proteins and standard (same known protein is better).
Rem : the solution should be put in the spectrophotometer just before reading, because continuous exposure to UV affects the signal.
Rem : One sample can be prepared during the incubation of the previous sample.
Rem : The duration of incubation could be sat up with the molecule of interest.

8- Plot a standard curve of amine detection, with the molar concentration of standard in x-axis and ODs on y-axis, then calculate for each sample the corresponding amine concentration.
Calculate the sample concentration taking in account the dilution factor.

Extracted from the complete protocol on OPA by (interchim, n.d.)[1]

[1] https://www.interchim.fr/ft/0/02727A.pdf

Appendix 2 – Solubilisation efficacy for gelatin

Solvent	% solubilised gelatin	SD
ChCl-OAD	66.08	3.07
CA-X-W	99.46	0.17
Water	33.20	2.06

The average solubilisation efficacy of gelatin for each solvent is presented with their standard deviation

Appendix 3 – Solubilisation efficacy for cellulose

Solvent	% solubilised cellulose	SD
ChCl-OAD	5.27	0.67
CA-X-W	4.79	0.75
Water	4.58	0.23

The average solubilisation efficacy of cellulose for each solvent is presented with their standard deviation

Appendix 4 – Refractive index of solvents and supernatants

Sample	Refractive index	SD
ChCl-OAD	1.46452	0.00005
CA-X-W	1.44016	0.00016
Water	1.33305	0.00000
ChCl-OAD – cellulose	1.46404	0.00016
CA-X-W – cellulose	1.43995	0.00142
Water – cellulose	1.33277	0.00001
ChCl-OAD – gelatin	1.46455	0.00004
CA-X-W – gelatin	1.44640	0.00171
Water – gelatin	1.33723	0.00047

The average refractive index is presented with standard deviations. The first three samples are the pure solvents, the next three are the solvent supernatants with solubilised cellulose, and the bottom three are the solvent supernatants with solubilised gelatin.

Appendix 5 – Calculations of theoretical RI of gelatin supernatant

RI for water was measured at 1.333, while RI for CA-X-W was measured at 1.440. The increase in RI from 1% dry matter to 65% dry matter was 8.03%.

$$\frac{1.440}{1.333} = 1.0803 \rightarrow 8.03\%$$

18.6 grams solvent was used, and a 65% dry matter would equate to 12.09 grams of dry matter. Adding the 1.4 grams of gelatin, the total dry matter would be 13.49 grams.

$$18.6 \times 0.65 = 12.09\ grams\ \ (+1.4\ grams\ gelatin = 13.49\ grams\ dry\ matter)$$

Since all the gelatin dissolved, the dry matter concentration of the supernatant would be 67.45%. Thus is the dry matter concentration of the supernatant 2.45% higher than the solvent

$$\frac{13.49}{20} \times 100 = 67.45\% \rightarrow 2.45\%\ higher\ dry\ matter\ than\ 65\%$$

The RI increase from 1% to 65% dry matter was 8.03%, making the RI increase 0.124% for each 1% increase in dry matter.

$$\frac{8.03\%}{65} = 0.124\%$$

If the RI increases 0.124% for 1% increase in dry matter, the RI will increase 0.304% from solvent to supernatant for a dry matter increase of 2.45%

$$0.124\% \times 2.45 = 0.304\%$$

If the RI should increase 0.304% from 1.440, the estimated RI of the gelatin supernatant of CA-X-W would be 1.444

$$1.440 \times 1.00304 = 1.444$$

Appendix 6 – Primary amino groups of gelatin supernatants given in mM

Supernatant	mM			SD
ChCl-OAD1	10.0	10.1	10.0	
ChCl-OAD2	11.9	11.7	11.8	1.3
ChCl-OAD3	13.3	13.3	12.9	
CA-X-W1	9.3	9.9	9.1	
CA-X-W2	9.5	9.4	9.3	0.8
CA-X-W3	10.7	11.2	11.0	
Water1	106.0	98.0	99.0	
Water2	7.7	6.9	7.5	0.9
Water3	5.8	5.2	5.8	

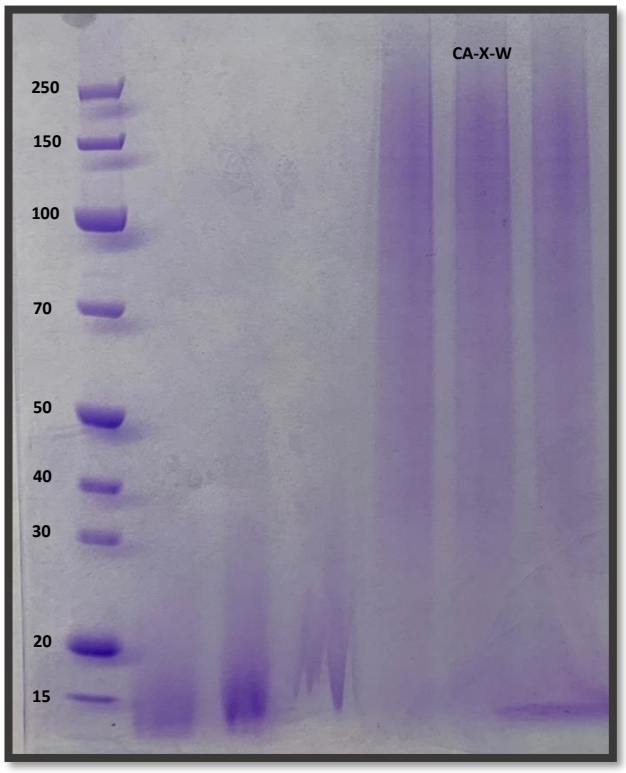
These values have been normalised to the same protein content using the values in appendix 7. The data for the first replication of gelatin solubilised in water (Water1) was not used for the graphic illustration in section 4.4.1 or the standard deviation and was therefore greyed out.

Appendix 7 – Protein content of gelatin supernatants given in mg/mL

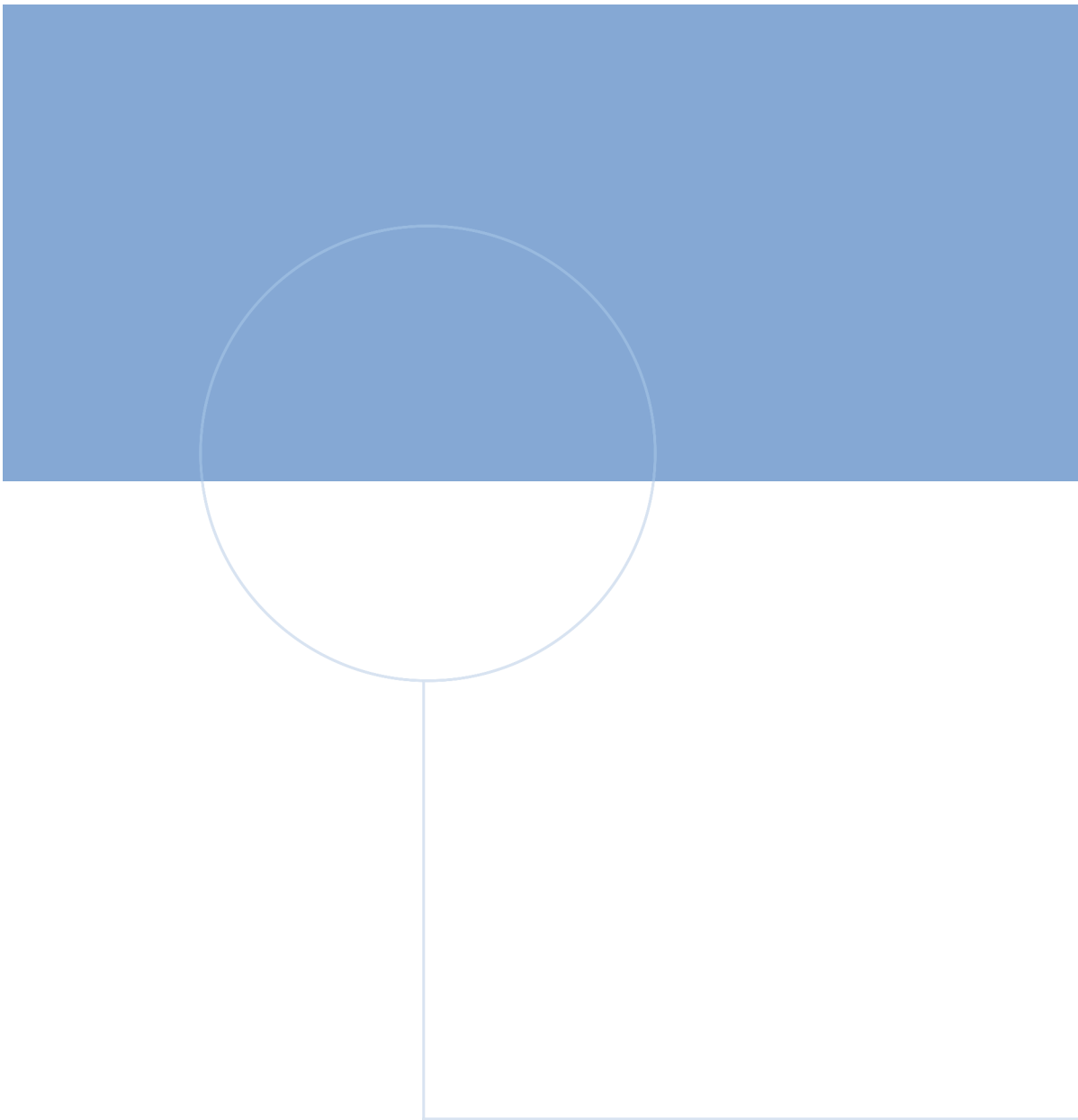
Supernatant	Protein mg/mL			SD
ChCl-OAD1	0.790	0.905	0.784	
ChCl-OAD2	0.817	0.601	0.696	0.081
ChCl-OAD3	0.729	0.729	0.709	
CA-X-W1	5.770	6.469	6.402	
CA-X-W2	6.808	6.086	6.537	0.362
CA-X-W3	6.244	6.875	6.920	
Water1	0.283	0.333	0.315	
Water2	1.339	1.483	1.425	0.100
Water3	1.195	1.258	1.267	

The data for the first replication of gelatin solubilised in water was not used for the graphic illustration in section 4.4.2 or the standard deviation and was therefore greyed out.

Appendix 8 – Protein content of gelatin supernatants given in mg/mL



The picture shows the gel from the first round of SDS-PAGE, the numbers marked on the standard indicated the kDa of the bands. The three wells to the far right are respectively CA-X-W1, CA-X-W2 and CA-X-W3. There can be observed to weak bands between 100 kDa and 150 kDa on all three replicates.



NTNU

Norwegian University of
Science and Technology