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Tuna Baydin

Optimization and Further Development of ConCordix Formulation Technology

Doctoral thesis

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
Philosophiae Doctor
Faculty of Natural Sciences
Department of Biotechnology and Food Science

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Trondheim, December 2022

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Tuna Baydin

Trondheim, September 5th of 2022

Preface

This doctoral dissertation is submitted in partial fulfilment of the requirements of the degree of Philosophiae Doctor (Ph.D.) at the Faculty of Natural Sciences of the Norwegian University of Science and Technology (NTNU). The research was conducted at the Department of Biotechnology and Food Science in Trondheim and Vitux AS in Oslo, Norway.

This industrial PhD project was funded by Vitux AS and the Research Council of Norway. The project started in March 2019 and the thesis was submitted in September 2022. In addition to the conducted research, the funding included the co-supervision of a master's project and thesis.

The main supervisor of my doctoral studies was Kurt I. Draget and the co-supervisor was Magnus N. Hattrem. Kurt I. Draget is a professor at NTNU and Magnus N. Hattrem is a senior researcher at Vitux AS.

This doctoral dissertation is a collection of scientific papers with the main research outcomes published in peer-reviewed scientific journals, a manuscript in preparation, and a patent application. The thesis guides the interested reader through the context and research methods of the conducted investigations.

Tuna Baydin
Trondheim, September 5^{th} of 2022

Summary

Food supplements are recommended for individuals lacking micronutrients in their diets, e.g., vegetarians, vegans, people with allergies, eating disorders, or bowel diseases. In addition, healthy individuals who are pregnant, breastfeeding, children, elderly, or do not have access to sufficient sunlight may need food supplements. Oral food supplements are commonly delivered in the form of tablets, hard or soft capsules, bulk oils (e.g., fish oil), or gummies. Many people have difficulties with swallowing tablets or capsules, whereas gummies generally contain a large amount of sugar and have a low payload. Liquid fish oils and gummies can also undergo oxidation after the packaging is opened. ConCordix (CCx) is a patented gelatin-based technology providing a user-friendly delivery form by combining water- and lipid-soluble active ingredients in a single dose protected from oxidation inside an airtight aluminum blister packaging. As CCx products have been manufactured and exported to different parts of the world, new climate zones and countries with various cultural backgrounds brought out new demands for the technology. The main three objectives of this PhD project were: i) to develop a more heat stable CCx for warmer climates, ii) to develop a plant-based alternative to classic gelatin-based CCx, and iii) to evaluate whether a higher bioavailability could be obtained for certain lipid-soluble actives.

The stability of gelatin-based CCx products may be compromised if units are exposed to high temperatures during transportation and storage. At high temperatures gelatin may degrade because of acid hydrolysis at its standard pH value, 4.5. Storage stability is also important for active ingredients in a CCx. Gelatin gels were prepared using two different Bloom value type B bovine and type A pig skin, as well as type A fish skin gelatin. The gels were stored at 20, 30, or 40 °C, and were analyzed using small amplitude oscillatory shear (SAOS) and size-exclusion chromatography coupled to online multi-angle light scattering (SEC-MALS) measurements at selected time points throughout their storage for up to 32 weeks. The results indicated that gels prepared with type A pig skin gelatins were more stable than fish skin gelatins, and type B bovine gelatin gels had the lowest stability. This was most likely due to type B gelatin gels being overall more negatively charged than type A gelatins due to the deamidation of asparagine and glutamine residues promoting proton condensation and acid hydrolysis. Higher Bloom values showed higher storage stability for gels prepared with both bovine and pig skin gelatin.

Ethical and ecological considerations have resulted in a search for mammalian gelatin alternatives. Fish gelatin can be obtained from cold or warm water species. As opposed to gelatins from cold water species, gelatins from warm water fish species exhibit more similar physical properties to mammalian gelatins. To improve the physical properties and storage stability of warm water fish gelatin gels co-solutes (sugar alcohols (sorbitol or xylitol) or sucrose) were included in formulations and followed for up to 16 weeks

of storage at three different storage temperatures (20, 30, or 40 °C). There was no statistically significant difference in the change of maximum dynamic storage modulus (G'), weight (M_w) or number average molecular weight (M_n) for different co-solutes. Sucrose gels showed browning at high storage temperatures, possibly due to the Maillard reaction of inverted sucrose. Fish gelatin solutions with sucrose exhibited higher viscosity than the gelatin solutions with sugar alcohols, which can lead to processability issues during manufacturing. As the sugar alcohol concentration increased, the stability of the gels increased, with significantly different changes in M_w.

There has been increase in the number of individuals following a vegetarian or vegan diet due to religious, health, ethical, and ecological considerations, resulting in a high demand for a plant-based CCx.Plant-based CCx were prepared using agar (with citrem or plant proteins as emulsifiers) and pectin (with lecithin as emulsifier) as gelling agents. The physical properties of agar (with citrem) and pectin formulations were characterized through water activity, syneresis, and SAOS measurements. Textural properties and *in vitro* lipolysis performances were also evaluated and compared to a traditional gelatin CCx. Pectin CCx had more similar melting point and texture to a gelatin CCx, compared to an agar CCx. The initial rate and extent of total *in vitro* lipolysis of the plant-based CCx were lower than the gelatin CCx. Agar CCx were also formulated using six plant proteins (pea, soy, rice, potato, faba bean, and chickpea protein) as emulsifiers. The emulsifying properties of the emulsifiers were studied at a CCx relevant pH and ionic strength. Heat stability of the CCx were studied before they were gelled using the thermal loop method. The results indicated that pea, chickpea, and faba bean proteins may have sufficient emulsifying properties in an agar CCx.

The lipid-soluble active ingredients in a CCx are pre-emulsified and may be taken up more quickly compared to if they were delivered in bulk oil. The droplet size of the emulsion is important since the lipases can only work at the interface. Therefore, investigating the impact of droplet size on the uptake kinetics of model compounds were of interest in this work. The lipolysis of three different droplet size emulsions (small, medium, or large) were studied using a simulated intestinal model *in vitro* and the uptake kinetics were studied *in vivo* in rats. *In vitro*, the lipolysis rate of the emulsions increased with decreasing droplet size. However, the medium emulsion had the highest rate of lipolysis per surface area. This may be due to a lower concentration of polysorbate 80 (PS80) in the medium emulsion than the small and large emulsions since higher concentrations of PS80 may have negatively affected the lipolysis rate for these two emulsions. For the *in vivo* studies, the rats were fed the same size emulsions loaded with vitamin D_3 (vitD_3) along with a bulk oil control. Plasma samples were collected from rats at selected time points up to 24 hours post feeding. The samples were analyzed using ultra-high performance liquid chromatography - mass spectrometry (UHPLC-MS) to detect vitD_3 and triacylglycerol (TAG) levels. For both of these molecules, the small and medium emulsions showed higher uptake than the large emulsion and the bulk oil control. However, only the TAG plasma values exhibited statistically significant differences.

Table of Contents

List of publications and patent applications

Contributions appended to the thesis

Paper 1: Baydin, T., O. A. Aarstad, M. J. Dille, M. N. Hattrem, and K. I. Draget (2022). "Long-term storage stability of type A and type B gelatin gels: The effect of Bloom strength and co-solutes". In: *Food Hydrocolloids* 127, pp. 107535. DOI: 10.1016/j.foodhyd.2022.107535.

Paper 2: Baydin, T., M. J. Dille, O. A. Aarstad, M. N. Hattrem, and K. I. Draget (2023). "The impact of sugar alcohols and sucrose on the physical properties, long-term storage stability, and processability of fish gelatin gels". In *Journal of Food Engineering* 341, pp. 111334. DOI: 10.1016/j.jfoodeng.2022.111334

Paper 3: Baydin, T., S. W. Arntsen, M. N. Hattrem, and K. I. Draget (2022). "Physical and functional properties of plant-based pre-emulsified chewable gels for the oral delivery of nutraceuticals". In *Applied Food Research* 2, 2, pp. 100225. DOI: 10.1016/j.afres.2022.100225

Paper 4: Dille, M. J., **T. Baydin**, K. A. Kristiansen, and K. I. Draget (2021). "The impact of emulsion droplet size on *in vitro* lipolysis rate and *in vivo* plasma uptake kinetics of triglycerides and vitamin D_3 in rats". In: *Food & Function* 12, pp. 3219-3232. DOI: 10.1039/d0fo03386c.

Paper 5: Baydin, T., M. N. Hattrem, and K. I. Draget (2022). "A comparative study of different plant proteins as potential emulsifiers in agar containing emulsions". Manuscript in preparation.

Patent application: Vitux Group AS (2022). "Chewable plant-based agar gel containing an oil-in-water emulsion and a plant-based macromolecular surfactant for the oral delivery of nutraceuticals and pharmaceuticals".

Contributions not appended to the thesis

Baydin, T., M. N. Hattrem, and K. I. Draget (2021). "The impact of sugar alcohols on the physical properties and short-term stability of fish gelatin". In: *Annual Transactions of the Nordic Rheology Society*, Vol. 29 (Online Conference).

Patent application: Vitux Group AS (2022). "Chewable plant-based agar gel containing an oil-in-water emulsion and a low molecular weight surfactant for the oral delivery of nutraceuticals and pharmaceuticals".

Patent application number: 2202754.4A
Applicant: Vitux Group AS
Filed in: United Kingdom
Filing date: 28 February 2022

The complete patent application could not be appended in this thesis since it is confidential until August 2023. Because of ongoing readjustments to the application, a summary of the invention could not be provided. Although the inventors of the patent are not disclosed yet, some of the work presented in this thesis contributed to the development of the invention.

List of abbreviations and symbols

CCx	=	ConCordix
DAG	=	Diacylglycerol
EAI	=	Emulsion activity index
ESI	=	Emulsion stability index
FA	=	Fatty acid
FDA	=	Food and Drug Administration
FFA	=	Free fatty acid
GI	=	Gastrointestinal
HLB	=	Hydrophilic-lipophilic balance
HMWE	=	High molecular weight emulsifier
IEP	=	Isoelectric point
LD	=	Laser diffraction particle analysis
LMWE	=	Low molecular weight emulsifier
MA	=	Malic acid
MAG	=	Monoacylglycerol
O/W	=	Oil-in-water
O/W/O	=	Oil-in-water-in-oil
PS80	=	Polysorbate 80
SEC-MALS	=	Size-Exclusion chromatography coupled to online multi-angle light scattering
SA	=	Sugar alcohol
SAOS	=	Small amplitude oscillatory shear
SGF	=	Simulated gastric fluid
SIF	=	Simulated intestinal fluid
TAG	=	Triacylglycerol
TCD	=	Trisodium citrate dihydrate
TPA	=	Texture profile analysis
UHPLC-MS	=	Ultra-high performance liquid chromatography - Mass spectrometry
vitD_3	=	Vitamin D_3
25-OH vitD_3	=	25-Hydroxyvitamin D_3
W/O	=	Water-in-oil
W/O/W	=	Water-in-oil-in-water
w/v	=	Weight by volume
w/w	=	Weight by weight

A	=	Area
a_w	=	Water activity
$D[4,3]$	=	Volume mean
$D[3,2]$	=	Surface mean
δ	=	Phase angle
η	=	Viscosity
E	=	Young's modulus
F	=	Force
γ	=	Shear strain
$\dot{\gamma}$	=	Shear rate
$G^{'}$	=	Dynamic storage modulus
$G^{'}_{max}$	=	Maximum shear storage modulus
$G^{''}$	=	Dynamic loss modulus
G^{*}	=	Complex modulus
I	=	Ionic strength
k	=	rate constant
M_n	=	Number average molecular weight
M_w	=	Weight average molecular weight
ΔP_L	=	Laplace pressure
σ	=	Stress
T_g	=	Gelling temperature
T_m	=	Melting temperature
T_t	=	Equilibrium sol-gel transition temperature

Chapter 1. Introduction

1. Context and motivation: ConCordix technology

Food supplements, i.e., dietary or nutritional supplements, are nutrition components consumed in addition to an individual's diet (Santini and Novellino 2017). Food supplements may be necessary due to dietary preferences and/or requirements, such as being vegan, vegetarian or pescatarian (Rizzo et al. 2016). People with allergies, eating disorders or gastrointestinal (GI) diseases may also need food supplements to complement their nutrition intake (Meyer et al. 2015; Reiter and Graves 2010). Moreover, there are healthy individuals who are recommended to take food supplements due to special circumstances. For instance, people over the age of 50 (Wood et al. 1995), pregnant and breastfeeding women (Moore and Parsons 2010), children (Wetzel and Wetzel 1939), and people with insufficient access to sunlight (Glerup et al. 2000) may be recommended to take different vitamins, minerals and fatty acids by the health authorities.

Food supplements are usually delivered orally in the form of tablets, hard/soft capsules, bulk oil, and powders (Jennings et al. 2008). These traditional delivery forms may result in compliance issues due to swallowing difficulties, especially in the pediatric and geriatric populations, as well as for patients suffering from dysphagia (Andersen et al. 1995; Wood et al. 1995). Fish oil is a popular dietary supplement due to a high concentration of omega-3 fatty acids and it is commonly consumed as bulk oil. Drinking long chain omega-3 fatty acids containing fish oil may not be appealing due to its strong taste and smell. In order to solve this problem, aromas are added to some products. In addition, after consuming products with fish oil, gastric reflux may occur causing unpleasant fish taste in the mouth throughout the day. Bulk oils are usually packed in bottles and once opened, the sample will be exposed to oxygen, promoting oxidation of the oil. This impacts the freshness of the oil and may result in rancid scent and taste. Oxidized oil may also have negative health effects (Dobarganes and Márquez-Ruiz 2003).

New chewable delivery forms in the form of gummies have been developed to overcome such compliance and taste issues. These products usually consist of gelatin, pectin and/or starch as the matrix generator and contain large amounts of sugar (Čižauskaitė et al. 2019). They have a low payload, i.e., they do not contain a high concentration of fish oil or other lipid-soluble active ingredients. Similar to fish oil bottles, gummies are usually sold in jars or bottles. Once the container is open, oxygen enters into the jar

and all the gummy units are exposed to oxygen, potentially resulting in the oxidation of active ingredients. Oxidation impacts the freshness of the unit and may cause changes in the taste of the gummy. Moreover, if the gummy jar is kept in a hot environment the gummies may melt and stick to each other.

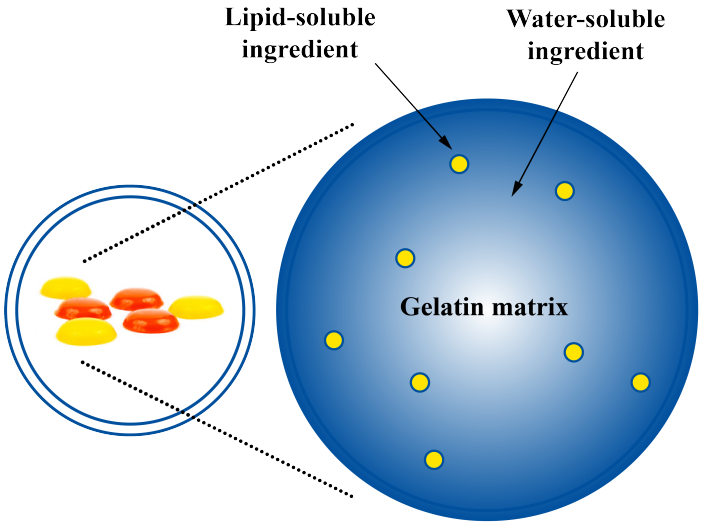

Figure 1: Water- and lipid-soluble ingredients in a CCx.

These issues have encouraged the development of an innovative delivery form, targeting the majority of the problems present with the traditional oral delivery forms and gummies. CCx is a patented delivery technology for the oral administration of dietary supplements manufactured by Vitux AS. This technology consists in encapsulating the active ingredients in a single dose chewable gel that provides a more user-friendly product compared to traditional tablets or capsules. A standard CCx product consists of a gelatin matrix which entraps pre-emulsified oil. CCx technology enables combining both water- and lipid-soluble ingredients dissolved in the aqueous and oil phases of the formulation, respectively (Fig. 1). A typical CCx is sugar free since it contains low-calorie sweeteners, i.e., polyols or sugar alcohols (SAs). It is also possible to obtain a high payload of lipid-soluble active ingredients in the formulation, making CCx a more robust delivery form compared to gummies. For example, with the CCx technology it is possible to obtain a product with fish oil potentially making up more than 50 (w/w)% of the dosage unit. In addition, each CCx unit is packed and sealed individually in aluminum blister packaging which prevents oxidation, prolonging the freshness of the product.

CCx technology has been developed by the Norwegian University of Science and Technology (NTNU) in collaboration with Vitux AS, and was patented in 2007 (PCT/GB2007/000261, filed January 25, 2007 designating the United States, and published in English as WO2007/085840 on August 2, 2007, which claims priority to Great Britain Patent Application No. 0601498.9 filed on January 25, 2006). Back then, Vitux AS was known as ProBio Nutraceuticals AS which then became Ayanda Group AS in 2009. Af-

ter that, the company took the name Vitux AS in 2016. Now, Vitux has approximately 80 employees with headquarters in Oslo and manufacturing sites in Andenes, Norway and Windsor, Canada. Vitux has been the third-party manufacturer of CCx technology for different customers, i.e., companies that offer food supplements in Europe, Asia, Australia, North and South America. Countries with at least one Vitux customer are shown in Fig. 2. A variety of formulations have been launched in the recent years with different oil types, flavors and active ingredients.

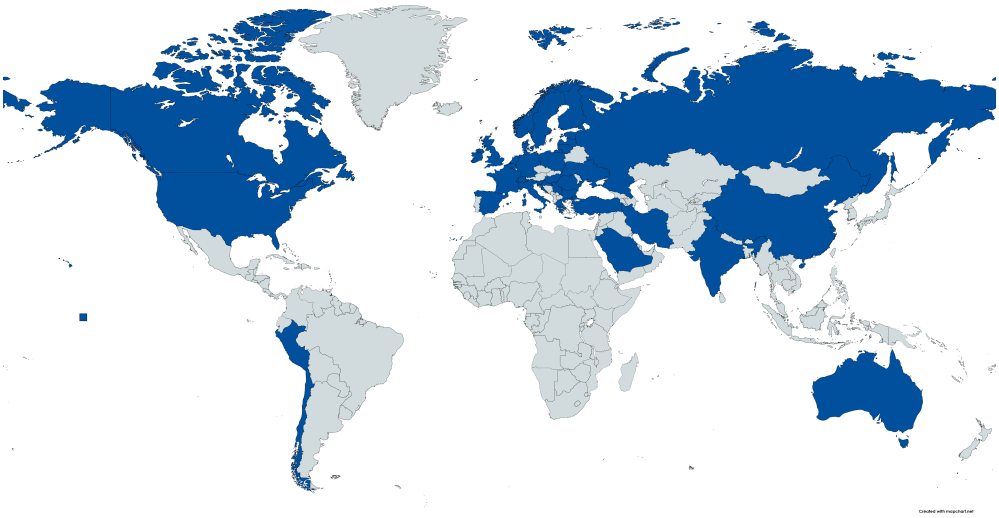

Figure 2: Countries with at least one Vitux customer.

With the expansion of customers all around the globe, optimization and further development of the CCx technology is needed in order to satisfy the demand in different parts of the world. The present industrial PhD project was initiated in line with these optimization and development requirements. In the next section, the objectives and scope of this thesis are presented.

2. Objectives and scope

The overarching hypothesis of this work was that by using gelatin with different properties or by using alternative matrix generators such as plat-based polysaccharides, by combining these biopolymers with different co-solutes, and by optimizing the droplet size will improve the stability, physical, and functional properties of chewable emulsion gels used for the oral delivery of nutraceuticals. The main objective of this thesis was to further develop and optimize the CCx technology to adapt products to new markets and related formulation challenges. Tropical and subtropical climates, as well as religious, ethical, and ecological considerations require the development of products which are more heat stable with ingredients having higher consumer acceptance and fewer restrictions in the market place. The research addresses the sub-goals presented in Fig. 3.

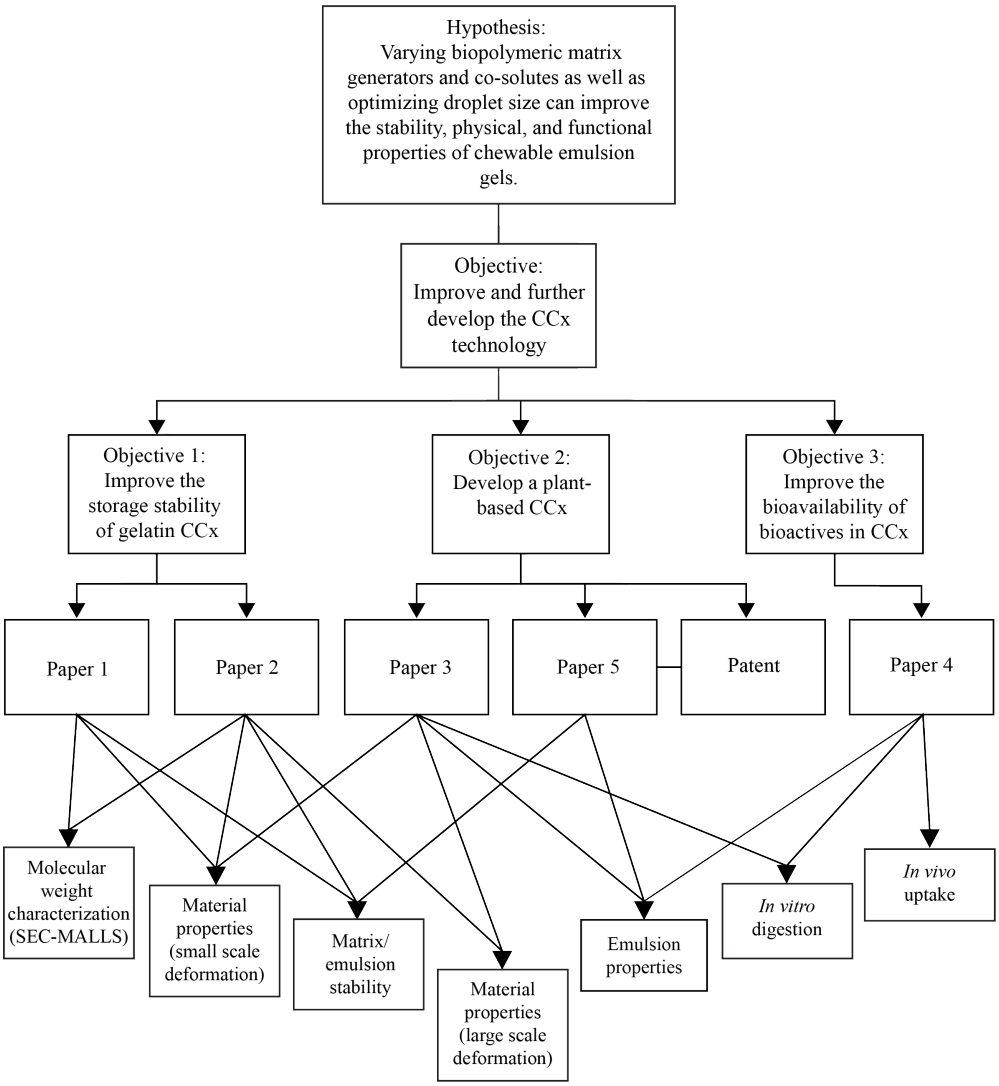

Figure 3: Hypothesis and objectives of the thesis with the organization chart of the papers.

The sub-goals are addressed through different papers which are connected to each other by the concepts given in Fig. 3.

2.1. Improvement of the storage stability of CCx products

For CCx, new market opportunities in warm climates create the challenge of thermal/heat/storage stability. Exposure to high temperatures during transportation and storage of the products affects the stability of a CCx. Especially in subtropical and tropical climate zones, the CCx may melt and/or stick to the packaging material. Exposure to high temperatures may cause faster degradation of gelatin through acid hydrolysis

and compromise the stability of the active ingredients. This may weaken gel strength and cause the gel to be very soft at the end of the shelf life of the product. CCx is dosed and sealed as individual chewable gels in aluminum packaging that protects the product from air and light. This provides good conditions for stability of the active ingredients. However, the product may stick to the foil due to melting/gelling cycles upon exposure to temperatures above 30 °C. This makes it difficult to get the whole unit out of the blister foil. Therefore, it is important to develop a CCx with a higher melting temperature (T_m).

Introducing another biopolymer with a higher T_m than gelatin may increase the heat stability of a CCx, but it also makes the system more complex to characterize. In this context, it is of interest to study the heat stability of gelatin from different sources such as bovine hides, pig skin, and fish skin. Other raw material and treatment factors such as pretreatment type and Bloom strength may be used to improve the stability of gelatin. In addition, the effect of co-solutes such as different SAs, sugars, and gum arabic on the stability of a CCx is of interest.

2.2. Development of a plant-based CCx

A standard CCx product is based on gelatin as gelling agent and emulsion stabilizer. Gelatin is a suitable matrix ingredient due to its elastic and chewy texture, emulsifier properties, T_m which provides a melt-in-the-mouth sensation, as well as gastric release. Gelatin is an animal-based biopolymer, obtained from the partial hydrolysis of connective tissues of cattle, pigs, fish, poultry etc.

In the recent years, there has been an increase in the number of individuals following a vegan or vegetarian diet in the Western world (Wozniak et al. 2020). This has lead to the demand of plant-based food alternatives in the market. Consequently, a major demand for a non-animal, i.e., vegan, CCx product has risen. Main reasons for following a plant-based diet are dietary, religious, or cultural requirements, and ecological, ethical, or health concerns. Developing a vegan CCx formulation requires changing the gelling agent from gelatin to a plant-based biopolymer (a polysaccharide). However, the majority of natural polysaccharides do not have surface active properties. Consumers have a negative perception of unnatural food containing chemically or enzymatically modified ingredients (Maphosa and Jideani 2018; Rozin 2005). Therefore, if a natural polysaccharide is to be used as the gelling agent in a plant-based CCx, addition of a separate emulsifier is required to stabilize the oil droplets. The choice of emulsifier should also be made with considerations of naturalness. Complexity of the system increases as new ingredients are added to the formulation. The natural polysaccharide gelling agent should work synergetically with the natural plant-based emulsifier in order to obtain a stable emulsion, which is a prerequisite for commercialization. In addition, an ideal plant-based CCx should satisfy mouthfeel. Since it is difficult to mimic the mouthfeel of a gelatin CCx, it was aimed to develop a plant-based CCx with an acceptable texture. Lastly, the vegan CCx should behave in the gastrointestinal tract as close to a traditional gelatin CCx as possible, to release the triacylglycerols (TAGs) and active lipid-soluble ingredients for optimal digestion and uptake.

2.3. Improvement of the bioavailability of lipid-soluble active ingredients in a CCx

Lipid-soluble active ingredients can be solubilized in a liquid oil, which further can be incorporated into CCx in the form of dispersed oil droplets immobilized in the gelatin matrix. The oil phase may contain the active ingredients of interest, e.g., lipid-soluble vitamins, Co-Q10, lutein or astaxanthin, or the oil itself may inherently contain fatty acids with physiological relevance. For example, fish oil contains high amounts of long chain omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that are well known to have beneficial health effects (Kris-Etherton et al. 2002). Oral omega-3 supplements traditionally come in the form of e.g., liquid cod liver oil or concentrated omega-3 in soft gel capsules. From literature it is known that emulsion-based delivery systems such as the CCx technology, may improve the uptake of fat-soluble active ingredients. In a previous clinical trial conducted by Vitux AS and the Department of Biotechnology at NTNU, an increase in the bioavailability of omega-3 fatty acids of approximately 44% was shown using CCx technology compared with a standard soft gel formulation (Haug et al. 2011).

Although, emulsions with a smaller oil droplet size have been reported to show higher bioavailability *in vivo* (Parthasarathi et al. 2016; Cho et al. 2014), the effect of droplet size on bioavailability and uptake of oils and active molecules is less clear than it is *in vitro*. Some previous *in vivo* studies show little or no benefit of smaller droplet size (Armand et al. 1999; Borel et al. 2001; Salvia-Trujillo et al. 2017). This motivates the investigation of how droplet size can affect the uptake of lipid-soluble active ingredients *in vitro* and *in vivo*. This would provide an opportunity of developing a CCx with optimized oil droplet size to increase the bioavailability of lipid-soluble active ingredients.

3. Organization of the thesis

This thesis is written as a collection of articles and it contains five appended articles, one patent application, and two introductory chapters. The current chapter introduces the context, motivation, objectives, and scope of this thesis. The upcoming sections introduce the main theoretical background. The next chapter presents a summary of the main conclusions from the scientific papers and suggests future perspectives that can be taken to continue the present work.

The articles in the thesis tackle three main objectives. The articles share common focus points and methodology as presented in Fig. 3. This industrial PhD project resulted in maintaining an industrial perspective while answering scientific questions. The first two papers contribute to the improvement of the storage stability of gelatin gels. The first paper sets a general evaluation of internal parameters (gelatin source, pretreatment type, and Bloom strength) of gelatin as well as different storage conditions on the stability of gelatin gels. The second paper focuses on fish gelatin and investigates the effect of different SAs and sucrose on its storage stability. It also questions the processability of the gels in a manufacturing scenario. Paper 3 and 5 explore plant-

based alternatives to a traditional gelatin-based CCx. The third paper compares two alternatives, agar and pectin, to gelatin as the gelling agent. The comparison focuses on physical properties and the functionality, i.e., *in vitro* lipolysis, of the systems. The fourth paper investigates the relationship between *in vitro* lipolysis and *in vivo* bioavailability of oil in a basic emulsion system. The fifth paper compares emulsifying properties of different plant protein emulsifiers in a simplified agar CCx.

4. Biopolymers and Biopolymer Gels

4.1. Biopolymers

Polymers are natural or synthetic macromolecules consisting of long, repeating chains of molecules (Hiemenz and Lodge 2007). Biopolymers are polymers with biological origin. For example, proteins are biopolymers composed of amino acid building units, and polysaccharides are composed of monosaccharides (Lehninger et al. 2005). Biopolymers have different functionalities depending on their structure. Polyelectrolytes carry multiple charges, anionic and cationic biopolymers carry only negative or positive charges, respectively, and neutral biopolymers do not carry a net charge (Vol'kenshtein 1970). Some biopolymers are gel forming, whereas some are not. Gel forming biopolymers are commonly referred to as hydrocolloids which are defined as a heterogeneous group of long chain polymers, i.e., proteins and polysaccharides, characterised by their ability to form viscous dispersions or gels when dispersed in water (Saha and Bhattacharya 2010) (Section 4.2).

A traditional CCx employs gelatin (Section 4.1.1) as the gelling agent (continuous matrix) and emulsifier. In the present work, two plant-based CCx were formulated using agar (Section 4.1.2) and pectin (Section 4.1.3) as gelling agents.

4.1.1. Gelatin

Gelatin, one of the most versatile biopolymers, has a wide range of applications in, e.g., confectionery, jelly desserts, soft or hard gel capsules, cosmetics and photography applications (Schrieber and Gareis 2007). It is a biopolymer obtained by the partial hydrolysis of collagen found in connective tissues, skin, bones, and tendons of animals. Pretreatment of collagen with acid or alkali results in type A and type B gelatin, respectively (Ward and Courts 1977). Type A gelatin is usually obtained from pig skin, fish, calf hides and bones or poultry, whereas type B gelatin is obtained from cattle hides and bones (Schrieber and Gareis 2007). In Europe, the majority (80%) of edible gelatin is sourced from pig skin, 15% is sourced from cattle skin, and the remainder 5% comprises porcine and bovine bones, poultry and fish (Boran and Regenstein 2010). Only less than 1.5% of gelatin is obtained from fish-derived sources worldwide (Karim and Bhat 2009).

When gelatin is dissolved in warm water, it forms random coil structures. As the solution is cooled down below the gelling temperature, denoted T_g, triple helical junction zones form. A gelatin gel is thermoreversible and when the gel is heated above the T_m, gelatin molecules re-form random coil structures (Fig. 4) (Schrieber and Gareis 2007).

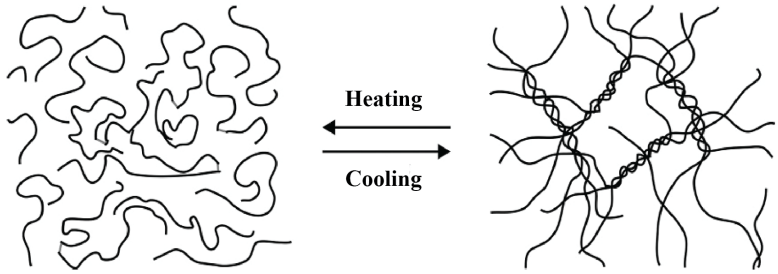

Figure 4: Gelling and melting mechanism of gelatin upon cooling and heating, respectively (Dille et al. 2021).

Gelatin is a protein, and as all proteins, it is composed of amino acids. The amino acid composition of gelatin depends on the source and pretreatment method being used (Ward and Courts 1977). For example, type A gelatin has in average 16 asparagine and 29 aspartic acid residues, whereas type B gelatin has 46 aspartic acid and no asparagine residues (per 1000 residues) (Dille et al. 2021). Glutamine and glutamic acid show a similar distribution for type A and B gelatins (Babel 1996). This is because of the deamidation of asparagine and glutamine under alkaline conditions, giving type B an increased number of negative charges (Rose 1987). As a result, the isoelectric point (IEP) of type A and type B gelatin are 8.5 and 5.0, respectively (Schrieber and Gareis 2007). IEP is the pH at which the protein has net zero electrostatic charge (McClements 2004a) (Subsection 5.1.2).

Imino acids (proline and hydroxyproline) play an important role in the formation of ordered structures and stabilization of triple helices (Derkach et al. 2020). As a result of relatively lower hydroxyproline and proline content in cold water fish gelatin compared to mammalian gelatin, the secondary structure of fish gelatin consists of more β-turn or β-sheet structures than triple helices (Derkach et al. 2019). Having a large number of β-structures is associated with lowered functional properties of fish gelatin such as a lower T_m and T_g (Karim and Bhat 2009). In addition, gelatin from cold water fish species have lower gel strength than warm water fish gelatins and mammalian gelatins (Gilsenan and Ross-Murphy 2000; Leuenberger 1991; Zhang et al. 2020). A comparison of gelatin from cold and warm water fish as well as from pork skin regarding their imino acid composition, gelling temperature (T_g), and melting temperature (T_m) are shown in Table 1.1.

Bloom strength is used to describe the rigidity of gelatin gels. It is measured by a standardized test called the Bloom standard test method in which a 6.67 (w/w)% gelatin solution is prepared and matured at 10 °C for 18 hours (Schrieber and Gareis 2007). The gel is then poked using a standard probe utilizing a texture analyzer. As opposed to mammalian and warm water fish gelatins, Bloom values can not be determined for cold water fish gelatins as they normally do not gel at 10 °C (Dille et al. 2021). However, with careful manufacturing it is possible to obtain cold water fish gelatins with Bloom values using a linear correlation between Bloom and dynamic storage modulus (G') (Eysturskard et al. 2009).

Table 1.1: Average imino acid composition, gelling temperature (T_g), and melting temperature (T_m) of pork skin, cold water, and warm water fish gelatin. The amount of hydroxyproline and proline residues are given as per 1000 total amino acids. T_g and T_m are average values reported for 10 (w/v)% solution with a cooling/heating rate 0.5 °C/min for non-specific species (Dille et al. 2021).

Origin	Species	Hydroxyproline	Proline	T_g (°C)	T_m (°C)
Mammalian gelatin	Pork skin	91	132	26.5	33.5
Warm water fish	Tilapia	79	119	21.5	28.5
Cold water fish	Cod	50	106	6.0	15.0

4.1.2. Agar

Agar refers to galactan polysaccharides occurring in red seaweeds specifically in Gelidiaceae, Gracilariacae, Phyllophoraceae, and Ceramiaceae species (Stanley 2006). Agars are linear polysaccharides consisting of alternating β(1,3)- and α(1,4)-linked galactose residues with repeating disaccharide units (Fig. 5). Agars consist of a neutral agarose and a charged agaropectin fraction (Armisen and Gaiatas 2009). The agarose fraction contains β-D-galactose and 3,6-anhydro-α-L-galactose units linked with glycosidic bonds (Helleur et al. 1985). The agarose fraction provides agar with its gelling properties, whereas the agaropectin fraction contributes little to the gel network (Nussinovitch 1997). Although agaropectin contains the same repeating units, some 3,6-anhydro-α-L-galactose moieties are replaced by L-galactose-6 sulphate, methoxy or pyruvate groups (Duckworth et al. 1971).

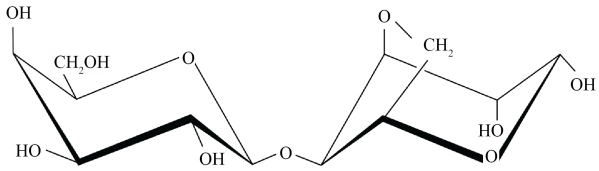

Figure 5: Repeating α(1,4)-linked disaccharide moieties of agar (Stanley 2006)

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Agar dissolves in water above 90 °C. When an agar solution is cooled below its T_g (between 32-45 °C), a coil-to-helix transformation followed by the aggregation of the helices results in the formation of a network structure through hydrogen bonding (Dai and Matsukawa 2012). As the gel is heated above its T_m (between 85-95 °C), the hydrogen bonds along the agarose chains break and a helix-to-coil transition results in gel melting. Agar gels exhibit large thermal hysteresis, i.e., difference between T_m and T_g (Sousa et al. 2021).

Agar gels exhibit syneresis, i.e., the expulsion of water from a gel (Armisen and Gaiatas 2009). Increasing agar concentrations result in lower syneresis (Banerjee and Bhattacharya 2011). Syneresis is usually considered as an undesirable characteristic since it may impact the stability and sensory characteristics of products, making them less appealing to consumers (Dille et al. 2021).

Agar is commonly used to prepare petri dishes for microbiology applications. In addition, agar gels are widely consumed as traditional food ingredients in Asia (Sousa et al. 2021).

4.1.3. Pectin

Pectins are a group of complex heteropolysaccharides present in the cell walls of terrestrial plants (Woodmansee 1948). Most commercial pectins are obtained from citrus peels and apple pomace (Zeeb et al. 2021). Although pectins show a high degree of variety in chemical composition, the main structural element of all pectins is a linear $\alpha(1,4)$-linked-D-galacturonic acid chain which forms a homogalacturonan backbone (Fig. 6A) (BeMiller 1986). If this backbone shows a high degree of methyl esterification, the backbone is referred to as highly methoxylated. Pectins are usually classified as high-methoxy ($>$ 50 %) and low-methoxy ($<$ 50 %) pectins, depending on the degree of esterification of the carboxyl group of the galacturonic acid-based units (Endress and Christensen 2009). Pectins are considered to consist of both smooth and hairy regions (De Vries et al. 1981). Smooth regions are responsible for forming cross-links and gel networks. Hairy regions consist of i) type I rhamnogalacturonans bound to galacturonic acid units, which carry a variety of sugars, e.g., arabinans and galactans, and ii) type II rhamnogalacturonans with complex highly branched sugars. The chemical structure of a pectin molecule is shown in Fig. 6B. Based on their chemical structures, pectins may have average molecular weights ranging between 50 - 150 kDa (Hua et al. 2015).

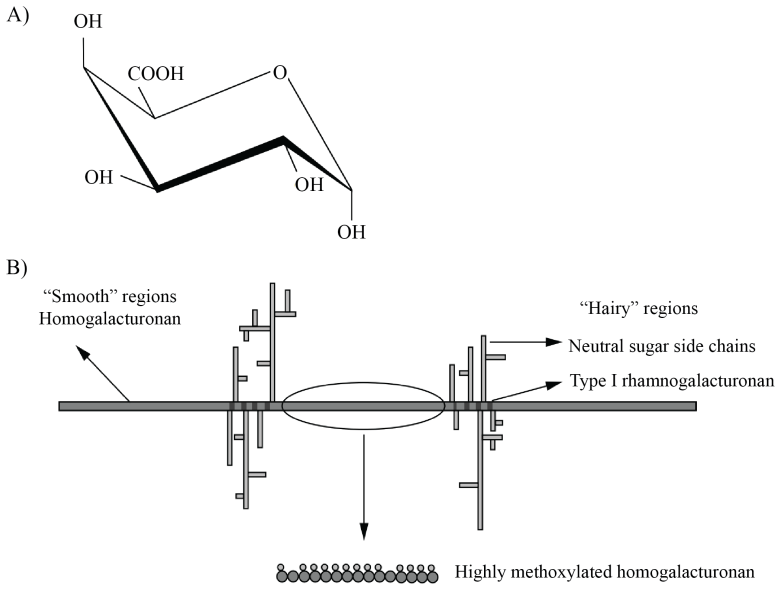


Figure 6: A) α-D-galacturonic acid unit and B) Schematic representation of a pectin chain. Modified from Ralet and Thibault 2002.

The gelation mechanism of a pectin solution depends on the methoxylation degree of

the pectin. Low-methoxy pectins gel in the presence of divalent ions, e.g., calcium, which bind to unmethylated and charged regions of homogalacturonan (Burey et al. 2008). The proposed gelation mechanism for low-methoxy pectins is called the egg-box model where divalent ions create cross-links between galacturonic acid residues, comparable to calcium binding between guluronate residues in alginate (Grant et al. 1973). Low-methoxy pectins may be amidated where 15 - 25% of the galacturonic residues contain primary amide groups (Da Silva and Rao 2006). Amidation increases pectin's reactivity to calcium, requiring a lesser amount of calcium for gelation (Lootens et al. 2003).

High-methoxy pectins gel at low pH (≈ 3) at which most acidic groups are protonated, and in the presence of high concentration of co-solutes such as sugars through the formation of cross-links between the methyl groups favored by hydrogen bonds and hydrophobic interactions (Axelos and Thibault 1991; Oakenfull 1991). Co-solutes lower the water activity (a_w) by changing the solvent structure through hydrogen bonding and hydrophobic interactions (Nussinovitch 1997). a_w is the ratio of partial vapor pressure of water in a system to the standard state partial vapor pressure of water at a specified temperature (Fontana 2000). It is a quantitative measure of the free water in a system.

Pectins are widely used in foods. Low-methoxy pectins are employed in the production of dairy, dietetic foods, and low-calorie products, whereas high-methoxy pectins are used in high sugar jellies, preserves, and jams (Da Silva and Rao 2006; May 1990).

4.2. Gels

A gel is a viscoelastic soft, solid, or semi-solid material, consisting of at least two components: one being a liquid and the other forming a three-dimensional cross-linked polymer or particle network (Almdal et al. 1993). If the liquid component of the gel is water, the gel is referred to as a hydrogel (Ross-Murphy and McEvoy 1986). The process of gel formation, i.e., formation of cross-links, is referred to as gelation.

In a gel, cross-links can be formed through covalent or hydrogen bonds, or electrostatic or hydrophobic interactions (Lu et al. 2018). The polymer segments of the molecule in between two cross-links are called elastic segments. The segment that continues out from a cross-link without being attached to a second cross-link is referred to as a loose end segment. Gelation of a hydrocolloid solution may be initiated by changes in temperature or pH, presence of ions, or through chemical or enzymatic reactions (Cao and Mezzenga 2020).

Gels have a wide variety of applications in foods, personal care products, biomedical technologies and drug delivery systems (Ahmed 2015). Examples of food gels are jelly desserts, butter, jams, sauces, and cheese (Saha and Bhattacharya 2010). Some food gels contain emulsions which are further described in Subsection 5.4.

4.3. Rheological characterization methods for viscoelastic materials

Rheology is the study of how materials flow responding to deformation. Flow properties of food define its structure during manufacturing, handling and mastication (Fischer

and Windhab 2011). The rheology of food materials can be studied using viscosity measurements, small strain deformation (Subsection 4.3.1), and large strain deformation (Subsection 4.3.2).

4.3.1. Small strain deformation

Newtonian liquids and Hookean solids are idealized representations of viscous liquid materials and elastic solid materials, respectively. Real liquids and solids are viscoelastic materials, which lie in between an ideal viscous and solid material, exhibiting solid- and liquid-like behaviour simultaneously.

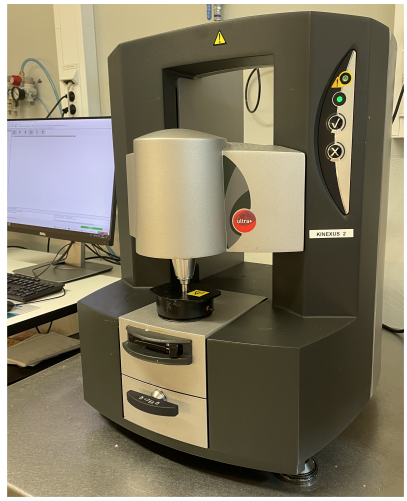


Figure 7: Photograph of Malvern Kinexus ultra+, the rheometer used in the present work.

Rheological properties of viscoelastic materials can be measured using a rotational rheometer (Fig. 7). SAOS measurements are performed by applying a harmonic oscillating deformation to the material, whereas viscosity measurements are preformed by applying a continuous deformation (Malkin and Isayev 2022).

Normal stress (σ), with the unit $\mathrm{N/m^2}$ or Pascal (Pa), is force, denoted F, perpendicularly applied to the material per unit area (A) with the unit $\mathrm{m^2}$ (Barnes et al. 1989):

$$\sigma = \frac{F}{A} \tag{1}$$

An F applied perpendicular to the material surface causes a relative deformation of this material, referred to as strain (ε). Strain is unitless and is defined as the ratio of change in length of a material (ΔL) to its original length (L_0) (Tabilo-Munizaga and Barbosa-Cánovas 2005):

$$\varepsilon = \frac{\Delta L}{L_0} \tag{2}$$

If F acts in parallel with the surface of the material, it is called shear stress (σ), resulting in shear strain (γ). Viscosity (η), with unit Pa $\cdot$ s, is defined as a material's

resistance to flow (Gage 1890). It is a result of loss of kinetic energy in a system, as a quantitative measure of internal friction when a material flows. η is the ratio of σ, with unit Pa, to shear rate ($\dot{\gamma}$) with unit s^{-1}, which is the rate of change in deformation, or strain, with time. η is calculated from the following equation (Barnes et al. 1989):

$$\eta = \frac{\sigma}{\dot{\gamma}} \tag{3}$$

The viscosity of a solution is dependent on several factors such as temperature, concentration of the material in solution, and molecular weight of the material. Elasticity is defined as a quantitative measure of the degree of structure within a material, i.e., the ability of a material to retain its shape after being deformed (Craig and Taleff 1996).

The viscosity of Newtonian liquids does not depend on $\dot{\gamma}$ or σ, with $\dot{\gamma}$ being linearly proportional to σ. Hookean solids have a linear relationship between σ and ε, and display perfect elastic behavior. For viscoelastic materials, if η decreases with increasing $\dot{\gamma}$, or η increases with increasing $\dot{\gamma}$, the material is referred to as exhibiting shear thinning, or shear thickening behavior, respectively (Picout and Ross-Murphy 2003).

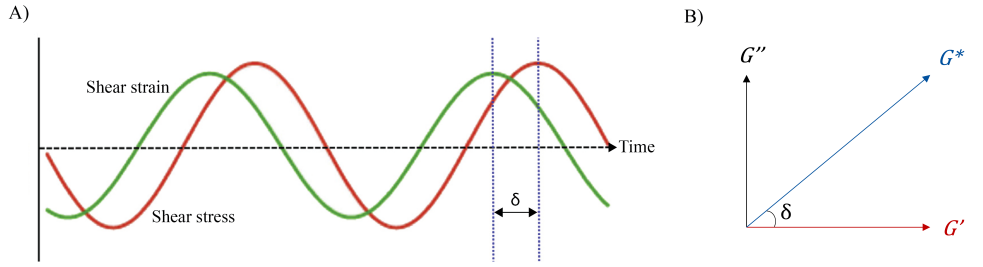


Figure 8: A) Sinusoidal curves of shear stress and shear strain. Modified from Chandran et al. 2020. B) The trigonometric relationship between complex modulus (G^*), dynamic storage modulus (G'), dynamic loss modulus (G''), and phase angle (δ).

The ratio of the maximum applied stress (σ_{max}) to maximum measured strain (γ_{max}) is the dynamic complex modulus (G^*) given in the following equation:

$$G^* = \frac{\sigma_{max}}{\gamma_{max}} \tag{4}$$

G^* is a quantitative measure of a material's rigidity, i.e., resistance to deformation.

Rotational rheometers can perform stress-controlled or strain-controlled measurements, where the resultant γ and σ is measured, respectively. As the oscillation continues, measured (or resultant) σ and γ form sinusoidal curves with time. The phase angle (δ) of the material is the lag phase between measured σ and γ (Fig. 8A). An ideal solid will have $\delta = 0°$, whereas an ideal liquid will have $\delta = 90°$. When a temperature ramp is applied, the transition from predominantly solid to liquid or vice versa occurs when $\delta = 45°$ (Malkin and Isayev 2022). This point corresponds to the T_m or T_g of a material. At this transition point, tan(δ) becomes frequency independent (Winter and Chambon 1986).

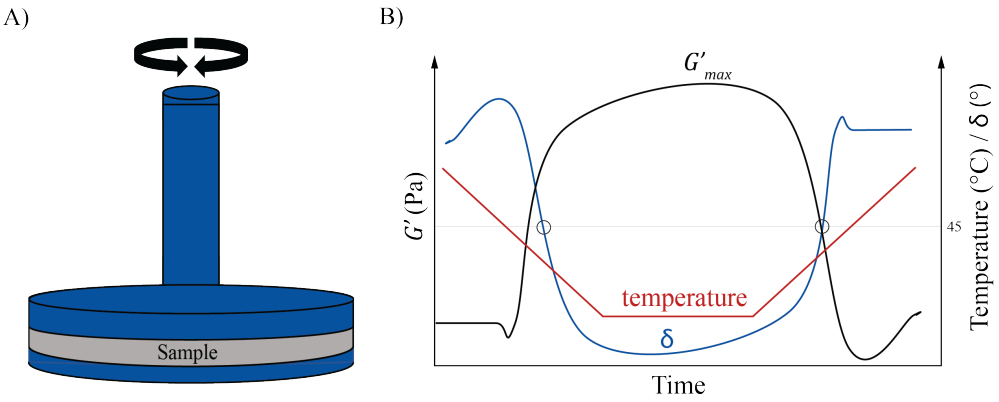


Figure 9: A) Schematic representation of an oscillatory measurement setup. B) An example curve from a small amplitude oscillatory shear measurement showing how shear modulus ($G^{'}$) and phase angle (δ) for the sample changes with temperature.

G^* has two components: i) $G^{'}$, and ii) dynamic loss modulus ($G^{''}$). $G^{'}$ is a measure of stored energy in a material with applied γ, $G^{''}$ is a measure of lost energy in a material with applied γ, representing viscous behavior (Barnes et al. 1989). $\tan(\delta)$ equals the ratio of $G^{''}$ and $G^{'}$ as shown in the following equation:

$$\tan(\delta) = \frac{G^{''}}{G^{'}} \tag{5}$$

The relationship between δ and G^* is shown in Fig. 8B.

For SAOS measurements, the material is placed between an oscillating circular probe and a stationary bottom plate (Fig. 9A). The rheometer is typically temperature controlled, allowing a thorough study of the rheological properties of a viscoelastic material under various conditions. Fig. 9B shows the gelling and then subsequent melting of a viscoelastic material. When δ is equal to 45° during gelling and melting, the T_g and T_m of the material can be estimated from the curve, respectively. Equilibrium sol-gel transition temperature (T_t) is the numerical average of T_m and T_g, defining the temperature at which the material transitions from primarily elastic to primarily viscous, and vice versa (Bohidar and Jena 1993).

4.3.2. Large strain deformation

Digestion starts with oral processing and texture is an important part of the sensory properties of food and other products aimed for oral delivery. Therefore, it is important to study sensory properties of new oral formulations (Lu 2013). Texture analysis is a type of large-scale deformation (large-strain test), providing an evaluation on the textural properties of a formulation which is to be handled and masticated. In the present work,

two different tests were performed using a texture analyzer (Fig. 10): i) single large strain compression, and ii) texture profile analysis (TPA).

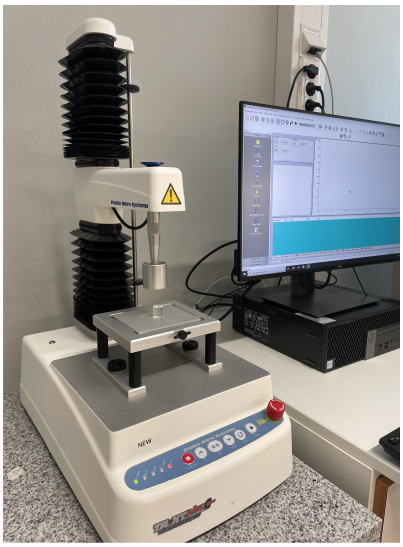


Figure 10: Photograph of Stable Micro Systems Ltd. TA.XT plusC Texture Analyser, used in the present work.

The single compression test deforms the sample using a standard size probe up to a certain strain, i.e., up to 75% of the gel height (75% strain), and then the probe retracts. The data is recorded by a software which enables the visualization of the compression parameters. Maximum force, with the unit of grams (g), and strain at failure (%) parameters are obtained from the fraction moment of the sample. Young's modulus (E) ($\mathrm{N/m^2}$) is calculated from the following equation:

$$E = \frac{\text{Gradient} \cdot \text{height of the gel}}{\text{Area of the gel}} \tag{6}$$

where gradient with the unit N/m is calculated by the ratio of force at linear initial part of the force/deformation curve. Area of the gel, with the unit $\mathrm{m^2}$, is the contact area of the gel with the probe which is calculated from the surface area of the uniform cylinder mold. E is a measure of the rigidity of a sample, whereas max force gives information about the hardness (Popov and Balan 1999). Strain at failure expresses the brittleness of the gel.

TPA is a standardized double compression test which aims to mimic the mastication of a food, e.g., gels. The test consists of compressing the gel once, retracting, and then compressing the gel again. Similar to the single compression test, the data is visualized and a TPA curve is obtained (Fig. 11). TPA parameters are calculated from the distances (d1 & d2) and areas under the curves (A1-4) marked in Fig. 11 (Bourne 1978):

- $Hardness$ (g) $= F1$
- $Adhesiveness$ (g $\cdot$ sec) $= A3$

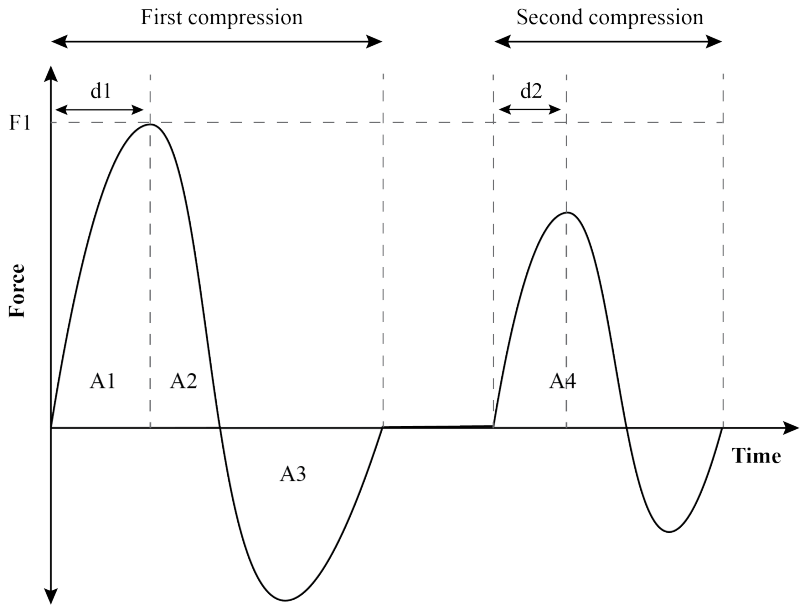


Figure 11: A typical texture profile analysis curve.

- $Resilience\,(\%) = A2/A1$
- $Cohesiveness\,(\%) = A4/(A1 + A2)$
- $Springiness\,(\%) = d1/d2$
- $Gumminess\,(\mathrm{g}) = F1 \cdot\ A4/(A1 + A2)$
- $Chewiness\,(\mathrm{g}) = F1 \cdot\ A4/(A1 + A2)\ \cdot\ d1/d2$

Chewiness and gumminess are comparable since gumminess is valid for semi-solid materials and chewiness is applicable for solids (Bourne 2002). More detailed information on texture analysis can be found in appended paper 3.

5. Emulsions

An emulsion is a mixture of two immiscible, i.e., non-mixing or non-soluble, liquids (McClements 2004a). It comprises two phases: i) continuous phase, and ii) dispersed phase. Usually, continuous and dispersed phases are either water or oil, and the interface region is in between the two phases (Fig. 14). If the oil phase is dispersed throughout the continuous aqueous phase, the emulsion is referred to as an oil-in-water (O/W) emulsion, e.g., milk and salad dressings. If the opposite occurs, the emulsion is referred to as a water-in-oil emulsion (W/O), e.g., butter and margarine. It is also possible to form double emulsions such as water-in-oil-in-water (W/O/W) or oil-in-water-in-oil (O/W/O) where the emulsion is dispersed in a second aqueous or oil phase, respectively (Garti 1997). The formation of an emulsion typically requires energy input which can be provided by homogenizers, e.g., high-speed mixers, colloid mills, high-pressure valve homogenizers, and ultrasonic homogenizers (McClements 2004a). In the present work, high-speed mixers were used as homogenizers (Fig. 12). To generate an emulsion, energy input is required. The need for energy input is due to fact that the interaction between the oil and water phase is thermodynamically unfavorable and thus, a smaller interfacial area is favorable (Walstra and Smulders 1998). Interfacial tension seeks to minimize the interfacial area and it is the constant of proportionality, i.e., the degree of imbalance of the molecular interactions, between the two immiscible liquids (McClements 2004a). The free energy change ($\Delta G_{formation}$) related to the formation of an emulsion is calculated from the following equation (Rajagopalan and Hiemenz 1997):

$$\Delta G_{formation} = Interfacial\ tension \cdot \Delta A \tag{7}$$

where ΔA is the change in the interfacial area. The interfacial tension generates a larger pressure inside the droplet which is referred to as the Laplace pressure (ΔP_L). It keeps a droplet in spherical shape, yielding the smallest surface area possible, and generates a larger pressure inside the droplet compared to the continuous phase. ΔP_L is calculated from Eq. (8) (McClements 2004a).

$$\Delta P_L = \frac{4 \cdot Interfacial\ tension}{d} \tag{8}$$

where d is is the diameter of the droplet. Hence, ΔP_L increases as d decreases. Reducing the interfacial tension facilitates the formation of smaller droplet size emulsions (Walstra and Smulders 1998). Emulsions are thermodynamically unstable systems and a mixture of oil and water will phase separate with time. Although they are not the focus of this work, it should be noted that there are thermodynamically stable emulsions such as self-microemulsifying drug delivery systems (Constantinides 1995). It is also possible to form kinetically stable emulsions that will be stable for a certain period of time by using stabilizers. Stabilizers increase the stability of an emulsion by either changing the rheological properties of the continuous phase, i.e., texture modifiers, or by forming a protective layer around the dispersed phase, i.e., emulsifiers or emulgents. Texture modifiers retard the movement of dispersed droplets by either increasing the viscosity

of the continuous phase, e.g., thickening agents, or by forming a gel of the continuous phase, e.g., gelling agents (Fig. 13) (McClements 2004a). The upcoming Section 5.1 focuses on emulsifiers.

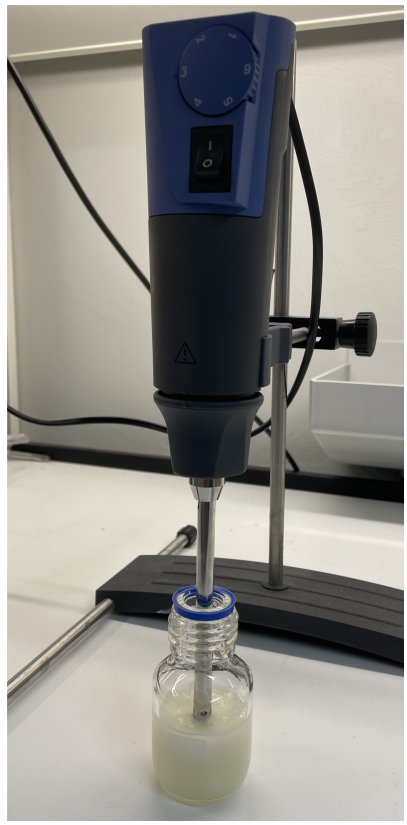


Figure 12: Photograph of IKA®T10 basic Ultra-Turrax®, one of the high-speed mixer homogenizers used in this work.

5.1. Emulsifiers

Emulsifiers are surface active molecules that are amphiphilic, i.e., containing both hydrophilic and hydrophobic domains. They reduce the required energy to generate an emulsion by promoting droplet disruption, adsorbing to the interface, and decreasing the interfacial tension between the two liquids (McClements 2004a). Food emulsifiers can be grouped as i) surfactants or low molecular weight emulsifiers (LMWEs), and ii) high molecular weight emulsifiers (HMWEs) (Fig 13). Bancroft's rule states that the phase in which an emulsifier is more soluble constitutes the continuous phase (Bancroft 2002). Therefore, it is possible to obtain O/W or W/O emulsions from two immiscible liquids depending on the type of the emulsifier being used. It should be noted that Bancroft's rule is not absolute, and it depends on other factors, i.e., amount of oil or water in the system (Holmberg et al. 2003). The hydrophilic-lipophilic balance (HLB) value of a

surfactant indicates if it is water-soluble (HLB > 9) or lipid-soluble (HLB < 7), being more suitable for a O/W emulsion or W/O emulsion, respectively (Griffin 1949).

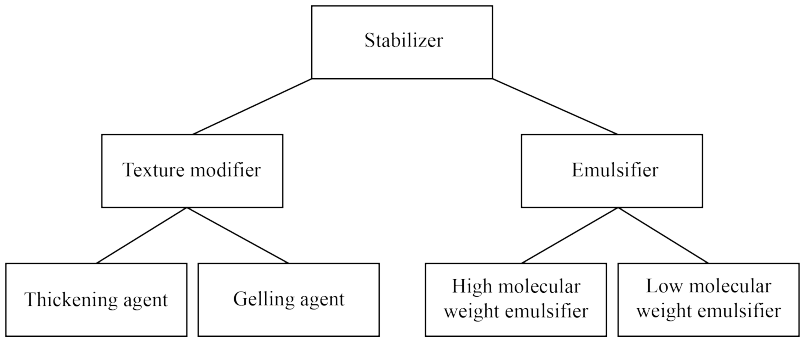


Figure 13: Stabilizers classified according to their functionality and molecular weight.

5.1.1. Low molecular weight emulsifiers

Surfactants are small molecules consisting of a hydrophilic polar head and a lipophilic non-polar tail group (Deffenbaugh 1994). They can be classified according to the electrostatic properties of the polar moiety such as cationic, anionic, non-ionic, and zwitterionic. Ionic surfactants stabilize emulsions by electrostatic repulsion, whereas non-ionic surfactants stabilize emulsions by steric repulsion (McClements 2004a).

Electrostatic repulsion emerges when all the droplets in an emulsion are stabilized by the same stabilizer, thus having the same net electrical charge which causes repulsion. Electrical double layer is the distribution of ions around a charged surface, e.g., the interface (Rajagopalan and Hiemenz 1997). Electrostatic stabilization is sensitive to changes in pH and ionic strength (I). The strength of electrostatic interactions also depends on separation distances between droplets and can be either long or short range. Long range electrostatic repulsion forms a repulsive energy barrier between droplets. More detail on this is given in Section 5.2. If the oil droplets are coated with a non-ionic stabilizer they are stabilized by steric repulsion. As two droplets approach each other closely, their stabilizer coatings overlap and interact, resulting in the compression of interfacial layers (McClements 2004a).

Common food surfactants are mono-glycerides (MAGs), di-glycerides (DAGs), lecithins (phospholipids), and polysorbates (Dickinson 1993; Tadros 2013). In the present work, three surfactants were used: PS80, citrem, and lecithin.

PS80, i.e., polyoxyethylene (20) sorbitan monooleate, is a synthetic LMWE. Being derived from polyethoxylated sorbitan, esterified with oleic acid, PS80 is non-ionic and water soluble. It forms stable O/W emulsions mostly unaffected by changes in pH, e.g., low pH in gastric conditions. PS80 has been approved as safe by the U.S. Food and Drug Administration (FDA 1977). It is commonly used in food, cosmetics, and pharmaceuticals (Goff et al. 1987; Nguyen 2018; Wang et al. 2021).

Citrem, i.e., monoglycerides citrate or citric acid esters of mono- and diglycerides, is a synthetic surfactant produced by reacting MAGs with citric acid or its anhydride in

the presence of an acid catalyst (Bade 1978). Citrem may contain minor proportions of free fatty acids (FFAs), free glycerol, free citric acid, MAGs, and DAGs (Fernández et al. 2020). It is anionic and water soluble (McClements 2004a). Citrem is generally recognized as safe by the U.S. FDA (FDA and Administration 1993) and is commonly used in the food industry, e.g., in beverages and infant formula (Amara et al. 2014).

Lecithin is a wide term used to describe groups of amphiphilic phospholipids in the cell membranes of living organisms, e.g., phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidic acid (Garti 1999; Hasenhuettl and Hartel 2008). It is mostly obtained from egg yolk, milk, soybeans, sunflower, rapeseed, or cottonseed. The hydrolyzed lecithin used in this project was obtained from sunflower. Commercial lecithins are by-products of vegetable oil production (Szuhaj 1983). Although their major components are phospholipids and TAGs, they also contain carbohydrates, glycolipids, free fatty acids, sterols, and other minor components of vegetable oils (Wendel 2000). Most phospholipids in lecithins are zwitterionic, i.e., the head group contains both positive and negative charges on the same molecule, and soluble in both water and oil (McClements 2004a). Since native lecithins have a low HLB value (typically ≈ 7) they have poor water solubility and may in some cases be sub-optimal for stabilizing O/W emulsions. However, through enzymatic partial hydrolyzation, i.e., forming lysophospholipids from phospholipids, they can be made more water soluble and better for O/W emulsions (Cabezas et al. 2016; Lilbæk et al. 2007). Lecithins are generally recognized as safe by the FDA and commonly used in the food and nutraceutical industry (FDA and Administration 1983; Shahidi 2005; Zeisel and Da Costa 2009).

5.1.2. High molecular weight emulsifiers

HMWEs are large molecues with surface active properties. Common food HMWEs are amphiphilic proteins, i.e., polymers of amino acids, and surface active polysaccharides, i.e., polymers of monosaccharides (Lehninger et al. 2005). Examples of surface active polysaccharides are chemically modified starches and cellulose, gum arabic, and high methoxylated pectins (Garti and Reichman 1993; Garti 1999; Yang et al. 2020). Protein emulsifiers can be obtained from milk, eggs, legumes, pulses, seeds, nuts, flours and juices (Donev 2015). In this project, six commercial plant proteins were used as emulsifiers in the agar CCx system: pea, soy, rice, potato, faba bean, and chickpea protein (chapter 2, paper 5). Commercial protein samples are usually fractions containing different proteins.

Proteins can stabilize emulsions by electrostatic repulsion and/or steric hindrance (Damodaran 2005; Lundgren and Ward 1949). The protein needs to carry a net charge in order to provide the former stabilization mechanism. The charge of the protein changes depending on the pH of the environment. If the pH is at the IEP, the protein is compact with net zero electrostatic charge (McClements 2004a). However, if the pH is sufficiently distant from the IEP, either lower or higher, than the IEP, the protein will carry a net charge which facilitates repulsive electrostatic interactions at the water-oil interface to stabilize the oil droplets (McClements 2004b). If the pH is distant from the IEP, sufficient intramolecular repulsion may cause the protein to unfold and expose hydrophobic domains which will interact with the oil droplets (Demetriades et al. 1997). The isoelectric

points of the proteins utilized in this project are given in Table 1.2.

Table 1.2: The commercial name, plant source, isoelectric point, and denaturation temperature of the proteins used in this project. The isoelectric point range is the average of all proteins typically present in these fractions, presented as given in literature. Although different subunits of proteins may have different denaturation temperatures, the given values are averages, presented as given in the literature. The references of this table can be found in appended paper 5.

Protein	Commercial name	Plant source	Isoelectric point	Denaturation temperature (°C)
Pea protein	Pisiva®	*Pisum sativum*	4.5 - 5.5	75 - 80
Soy protein	SUPRO® 595	*Glycine max*	4 - 5	> 90
Rice protein	Ortiva®	*Oryza sativa*	4.5 - 5	> 70
Potato protein	Solanic® 200	*Solanum tuberosum*	4 - 6	> 60
Faba bean protein	Faba-tein™	*Vicia faba*	5 - 5.5	> 85
Chickpea protein	-	*Cicer arietinum*	4 -5	> 90

The conformation of the proteins is also affected by the ionic strength, I. At high I, the electrostatic repulsion between the dispersed oil droplets is reduced, whereas at low I, electrostatic repulsion between the oil droplets is increased (Ghosh and Bull 1963). The solubility of proteins also change with I. As a general rule, the solubility of proteins increase at low I, whereas the opposite is observed at high I. This is referred to as "salting-in" and "salting-out" effect, respectively (Arakawa and Timasheff 1984). These two effects impact the emulsifying properties of proteins (Ettoumi et al. 2016).

Protein higher order structures, i.e., tertiary and quaternary structure, are highly dependent on temperature since proteins may denature under high temperature (Kelly et al. 1997). Denaturation is an irreversible process that may result in the loss of function of the protein, e.g., surface activity. Denaturation may also be caused by high/low pH or high I (Hasenhuettl and Hartel 2008).

5.2. Destabilization mechanisms

Emulsions are dynamic systems. The dispersed droplets continuously move and collide with each other due to Brownian motion. Since emulsions are thermodynamically unstable they will eventually destabilize, although it could take hours, weeks or even years depending on the level of kinetic stabilization (McClements 2004a). Potential destabilization mechanisms of a kinetically stable O/W emulsion are shown in Fig. 14.

Sedimentation and creaming may occur due to the gravitational separation of the two phases. If the density of the continuous phase is higher than the dispersed phase, creaming may occur (Dickinson 1994). The opposite would result in sedimentation. Larger droplets are more susceptible to creaming than smaller droplets since droplet movement is dominated by gravity for larger emulsion droplets (McClements 2011). Gravitational separation causes the oil droplets to come into close contact, which may promote flocculation or coalescence (McClements 2004a).

Flocculation occurs if dispersed oil droplets aggregate without merging together, i.e., forming flocs. Steric and electrostatic stabilization of the droplets may result in weak and strong flocculation, respectively (Hunter 1986). Weak flocculation is reversible if temporary shear forces, e.g., shaking, are applied, whereas strong flocculation is irreversible (Dickinson 1994). Strong flocculation occurs when long range electrostatic repulsion between the droplets is overcome by attractive van der Waals interactions at short distances. The formation of flocs may increase the rate of gravitational separation (McClements 2004a).

Depletion flocculation may occur if there is a high concentration of colloidal particles, such as surfactant micelles or biopolymers, present in the continuous phase. These colloidal particles may cause attractive interactions between oil droplets, overcoming the repulsive interactions. The attractive interactions are caused by the exclusion of the colloidal particles from a narrow region around the droplets called depletion zone (McClements 2004a). The osmotic pressure difference between the depletion zone and the continuous phase promotes droplet aggregation (Dickinson 1988). Bridging flocculation occurs when a charged ion or biopolymer binds to oppositely charged oil droplets, physically keeping them together. This type of flocculation may occur if a biopolymer is used as the emulsifier and the concentration of the biopolymer is insufficient to stabilize all individual droplets (Dickinson et al. 2003).

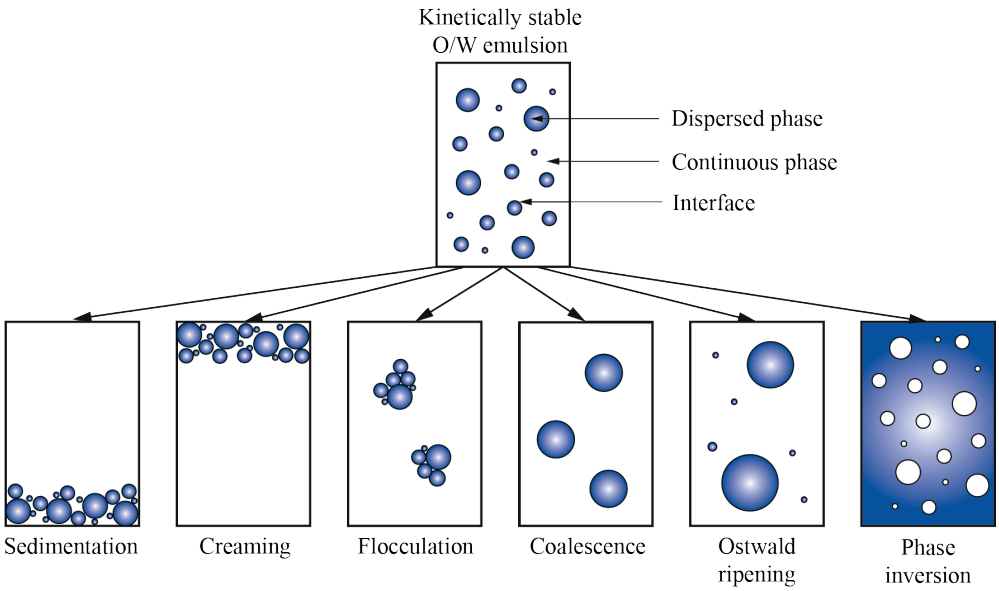


Figure 14: Destabilization mechanisms of a kinetically stable oil-in-water emulsion. Modified from McClements 2004a.

Coalescence occurs if two dispersed oil droplets merge together to form larger oil droplets. Coalescence decreases the total surface area of the emulsion (ΔA) which decreases $\Delta G_{formation}$, making the the emulsion more thermodynamically favorable (Eq. (7)). Coalescence is irreversible and it may be the first step leading to bulk phase separation

(McClements 2004a).

Ostwald ripening occurs when the dispersed phase has partial solubility in the continuous phase. Large droplets grow at the expense of small droplets. The solubility of the dispersed phase increases as the droplets get smaller (Kabal'Nov et al. 1987). It is more common for polydisperse emulsions to exhibit Ostwald ripening (McClements 2004a).

Phase inversion occurs if an O/W emulsion changes into a W/O emulsion or vice versa. Usually this process is triggered by changes in the composition or environmental factors such as emulsifier concentration and temperature (Dickinson 1994). Phase inversion is utilized during the manufacturing of some food systems, e.g., butter and margarine. However, it is undesirable for most food systems since it results in changes in the texture, stability, and taste of the emulsion (McClements 2004a).

5.3. Droplet size analyses

To characterize and study the stability of an emulsion, droplet size analyses are essential because droplet size influences the stability of an emulsion and potentially its functionality. An emulsion may be monodisperse or polydisperse, meaning it may contain droplets of the same size or varying size, respectively. Laser diffraction particle analysis (low angle laser light scattering, LD), dynamic light scattering, coulter counting, ultrasonic measurements, nuclear magnetic resonance, and electron microscopy are methods for measuring the droplet size of an emulsion (McClements 2004a). In the present work, LD was used to determine the droplet size of the emulsions (Fig. 15).

Figure 15: Photograph of the Malvern Mastersizer Hydro®3000 that was utilized for laser diffraction particle analyses in the present work.

LD measures the scattered light as a laser beam (the light source) passes through a dispersed sample. The intensity and scattering of the measured light is measured by a diffraction system as a function of scattering angle, generating a diffraction pattern. This pattern changes depending on the size of the particles, e.g., oil droplets in an O/W emulsion. Smaller droplets scatter light at wider angles, whereas larger droplets scatter light at narrower angles (Latimer and Pyle 1972). When the laser beam hits the droplets,

the light is scattered and detected at different angles. If a light source with more than one wavelength is used, detection in a wider size range can be obtained, providing more precise results (Rawle 2003).

The volume mean ($D[4,3]$) and surface mean ($D[3,2]$) are commonly used parameters defining the droplet size of an emulsion. $D[4,3]$ is associated with the size of the particles that make up the bulk of the sample volume. It is calculated from the following equation (Hunter 1986):

$$D[4,3] = \frac{\Sigma {D_i}^4 \cdot n_i}{\Sigma {D_i}^3 \cdot n_i} \tag{9}$$

where D_i is the diameter of a particle and n_i is the number of particles. $D[3,2]$ is related to the average surface area of droplets that are exposed to the continuous phase per unit volume of emulsion. In other words, it is the diameter of a droplet having the same volume/surface area ratio as the whole emulsion. It is calculated from the following equation (Walstra 2003):

$$D[3,2] = \frac{\Sigma {D_i}^3 \cdot n_i}{\Sigma {D_i}^2 \cdot n_i} \tag{10}$$

$D[4,3]$ is more sensitive than $D[3,2]$ to the presence of large particles in the size distribution. If the droplet size measurement of an emulsion gives similar $D[4,3]$ and $D[3,2]$ values, the emulsion exhibits a monodisperse distribution. Dissimilar $D[4,3]$ and $D[3,2]$ values indicate a polydisperse emulsion. Flocculation, coalescence, and Ostwald ripening result in larger droplet size averages as a function of time (McClements 2004a).

5.4. Emulsion gels

Emulsion gels, also referred to as gelled emulsions or emulgels, are complex soft-solid colloidal materials that contain an emulsion and a gel (Dickinson 2012). They can be classified as emulsion droplet-filled gels or emulsion droplet-aggregated gels (Lin et al. 2020) as depicted in Fig. 16. Emulsion droplet-aggregated gels consist of aggregated emulsion droplets, forming a network structure which disrupts the gel matrix. Alternatively, the emulsion droplets may create the gel matrix. Emulsion droplet-filled gels consist of a continuous gel matrix which encapsulates the embedded emulsion droplets. The emulsion gels made in this project are emulsion droplet-filled gels.

If the emulsion droplets have strong attractive interactions with the surrounding gel network, the droplets are referred to as active fillers (Tolstoguzov 1996). On the other hand, if the emulsion droplets have little to no interaction with the surrounding gel network, the term inactive filler is used (Chen and Dickinson 1999; Ring and Stainsby 1982). Gelatin emulsion gels usually exhibit an active filler effect, whereas polysaccharide emulsion gels prepared with hydrocolloids with low surface activity typically exhibit an inactive filler effect (Koç et al. 2019). For active fillers, the inclusion of sufficiently small oil droplets increases the elastic properties of the emulsion gel such as the dynamic storage modulus, compared to the gel alone. On the contrary, inactive fillers usually decrease the elastic properties of the emulsion gel compared to the gel alone (Ring and

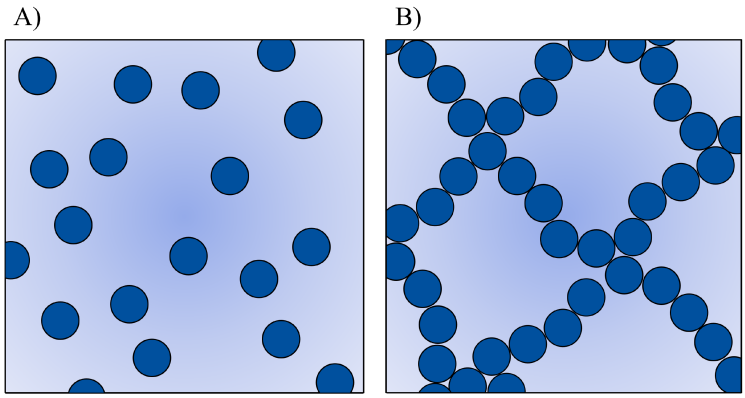


Figure 16: Schematic representation of A) emulsion droplet-filled gels B) emulsion droplet-aggregated gels.

Stainsby 1982). Active fillers may be beneficial for the stability of the emulsion gel due to droplets being arrested in the gel matrix while inactive fillers may lead to the long-term destabilization of the emulsion.

Emulsion gels are used in food, pharmaceuticals, and cosmetics (Farjami and Madadlou 2019). Real food emulsion gels are complex systems in which oil droplets may have an ambiguous filler role (Chen and Dickinson 1999). In addition, for emulsion gel systems consisting of one or more polymers and surfactants, the rheological character of the system becomes more complex since each component may contribute to rheological properties (Farjami and Madadlou 2019).

5.5. Lipolysis

When dietary fats are consumed they are in the form of TAGs, i.e., fully acylated glycerol derivatives which are composed of three fatty acids (FAs) esterified to a glycerol backbone (Silverthorn 2013) and phospholipids. If the glycerol backbone of a TAG is linked to one or two FAs, the molecule is referred to as a MAG or DAG, respectively. Lipolysis is the hydrolytic cleavage (or, in other words, the breakdown) of the ester bonds in TAGs to yield glycerol and FFAs. Depending on the location of lipolysis, DAGs and MAGs are also produced (Armand 2007; Mu and Høy 2004).

Upon consumption of a fat containing food, the first step of GI digestion is oral processing through mastication. Although humans have small amounts of lingual lipases secreted along with saliva by serous lingual glands, they have little physiological function (Feher 2017). In some animals such as rodents, lingual lipases contribute to the digestion of up to 30% of the ingested lipids (Hamosh et al. 1979). After mastication, the food is swallowed and it reaches the stomach where it is subjected to a low pH and high I environment. Muscle contractions of the stomach provide shear forces contributing to the emulsification of fats (Carey et al. 1983). The food is exposed to gastric lipases secreted

by the gastric mucosa. Gastric lipases break down TAGs into DAGs and free fatty acids. In humans, 10 – 30% of total lipolysis in the GI tract takes place in the stomach. In contrast, rats do not have gastric lipase activity, although their lingual lipases are also active in the stomach (DeNigris et al. 1988; Hamosh, Scow, et al. 1973; Phan and Tso 2001). Digestion products such as FFAs may displace emulsifiers and cause emulsion destabilization of pre-emulsified oils (Infantes-Garcia et al. 2021). Another digestive enzyme in the stomach is pepsin, which breaks down proteins. The gastric stability of protein-stabilized emulsions in the stomach may be compromised due to the presence of this enzyme (Sarkar et al. 2009). Surfactants such as PS80 may inhibit the adsorption of gastric lipases to interface, reducing the extent of lipolysis (Infantes-Garcia et al. 2021).

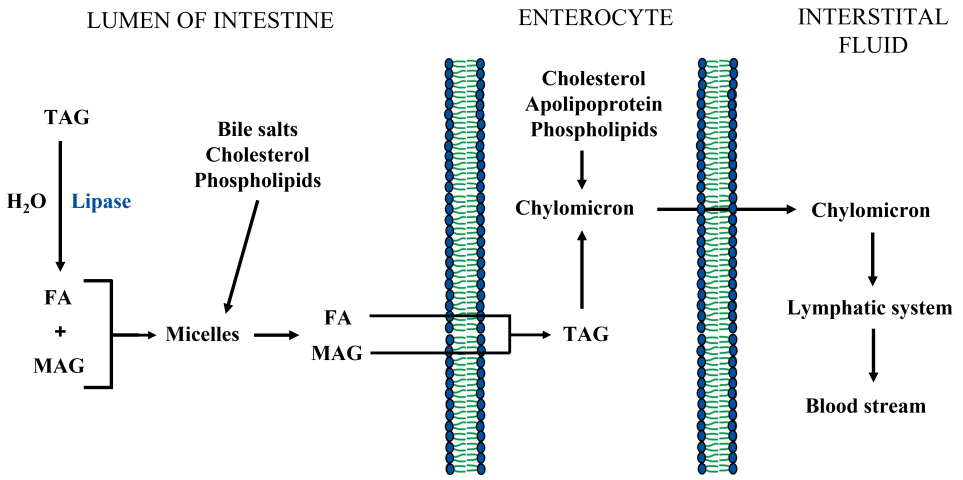


Figure 17: Illustration of the journey of a triacylglycerol molecule from intestinal lumen to blood stream. Modified from Berg et al. 2010.

The contents of the stomach are transferred to the duodenum, which is the first part of the small intestine, through gastric emptying. Gastric emptying is regulated partly by cholecystokinin, a hormone secreted upon the presence of fatty acids or protein digestion products in the duodenum. When ingested lipids reach the duodenum, mechanical contractions result in smaller emulsion droplets, leading to increased surface area. In the duodenum, TAGs and other digestion products encounter pancreatic lipase, which can only work on the oil-water interface (Mun et al. 2007; Senior 1964). Phospholipids are digested by phospholipases, primarily by phospholipase A_2 (Ihse and Arnesjö 1973). A TAG is hydrolyzed into an FFA and a DAG which is consecutively hydrolyzed into a MAG and an FFA. Pancreatic lipase requires bile salts and colipase for optimal enzymatic activity. After entering the duodenum, bile mixes with FFAs and MAGs. This mixture along with other surface active components such as phospholipids, form micelles in the lumen of the intestine above the critical micellar concentration (Hofmann 1963). Micelles carry the final digestive products, i.e., MAGs and FFAs, to the intestinal epithelial cells (enterocytes). Micelles are small enough to diffuse through the mucosa to the narrow areas between the microvilli which covers the intestinal wall and increases the

total absorptive surface area of the small intestine (Caspary 1992). Micelle formation is a dynamic process in which micelles disintegrate and reform constantly. When micelles bring the final digestive products in close proximity to the enterocyte wall, MAGs and FFAs leave the micelles and diffuse into the enterocytes where they reassemble to form TAGs in the endoplasmic reticulum (Silverthorn 2013). TAGs are then packaged into chylomicrons, i.e., lipoprotein-transport particles, which contain the TAGs in the core of the particle and have an outer shell made up of cholesterol, phospholipids, and apolipoproteins (Schaefer et al. 1978). Chylomicrons transport TAGs outside of enterocytes through exocytosis. Chylomicrons carry TAGs with long-chain FAs, whereas short- and medium-chain FAs are primarily transported to the liver through the portal vein to be metabolized (Papamandjaris et al. 1998). As chylomicrons omit the first pass metabolism of the liver and enter the lymphatic capillaries, they are transported to the thoracic duct where they enter the blood stream to be carried throughout the different tissues of the body (Fig. 17). The lipolytic products can be taken up by muscle or adipose cells for energy production or storage, respectively (Mu and Høy 2004).

5.5.1. In vitro lipolysis

Studying the lipolysis of new oral formulations in clinical trials and *in vivo* systems are costly, time consuming, and require tedious but necessary ethical approvals. In this context, *in vitro* lipolysis experiments that mimic human GI physiology provide preliminary evaluation of the lipolysis of different formulations (Brodkorb et al. 2019).

It is important to design *in vitro* lipolysis experiments with physiological relevance. Different compartments of the GI tract have particular conditions such as temperature, I, presence of enzymes, and salts. Due to the complexity of human physiology, it is not possible to precisely simulate *in vivo* conditions with *in vitro* setups. The aim of *in vitro* experiments is to obtain physiologically representative experimental conditions. Although oral processing of food can be simulated in great complexity (Panda et al. 2020), inclusion of the gastric and intestinal stage in static *in vitro* lipolysis is recommended (Brodkorb et al. 2019). This recommendation is followed in the present work. Static *in vitro* models cannot mimic the complex dynamics of digestion, e.g., they do not model different components of the intestine (Eldemnawy et al. 2015). However, there are dynamic *in vitro* models that attempt to simulate the dynamic nature of the GI tract (Butler et al. 2019).

The standard recommended temperature of a lipolysis medium is 37 °C (Hur et al. 2011). The pH throughout the GI tract varies for different organs and also for fed and fasted state. Although the pH of the human gastric juice is 1.5 - 2.0, most gastric *in vitro* models employ pH values between 1.4 - 3.0 (Fujimori 2020; Sams et al. 2016). The recommended pH interval for the simulated intestinal fluid (SIF) is 6.5 - 8.5 (Larsen et al. 2011). The lipase source is another important component of *in vitro* lipolysis models. Although microbial lipases are commonly used to replace human gastric enzymes, their equivalence is questionable (Sams et al. 2016). Rabbit gastric extract (used in this work) has been suggested as a suitable replacement (Brodkorb et al. 2019). RGE also contains pepsin. For intestinal models, porcine pancreatin (also used in this project) has been

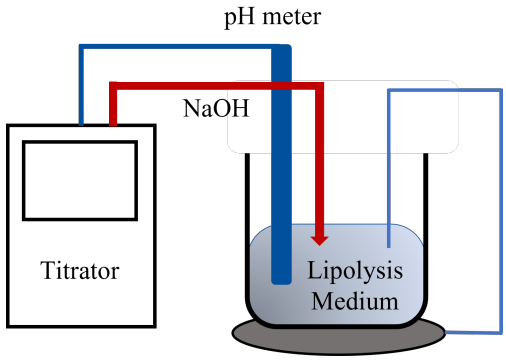


Figure 18: A schematic representation of the in vitro lipolysis set up used in this project.

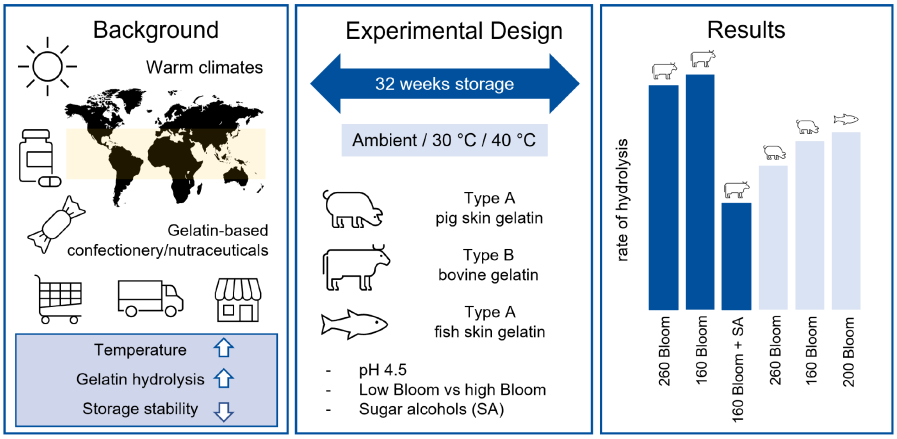
recommended since it is a lipase source that also contains other pancreatic enzymes such as phospholipase A_2, colipase, cholesterol esterase, and TAG lipase (Larsen et al. 2011). The lipolysis medium also contains bile acids, phospholipids, and calcium. Bile acids and calcium aid the removal of FFAs from the lipolysis medium since FFAs can accumulate on the interface, preventing the access of lipases to the surface of oil droplets (McClements and Li 2010). This happens because of the static nature of the *in vitro* lipolysis model. Bile acids also help against the accumulation of surfactants at the interface, preventing lipase access (Wilde and Chu 2011).

Many contemporary *in vitro* lipolysis models utilize the standardized pH-stat method, which correlates the decrease in pH with the release of FFAs from TAGs (Li et al. 2011). The pH of the SIF is monitored by a pH meter as the lipolysis reaction proceeds. The pH meter is connected to a titrator which maintains the pH of the SIF, e.g., at pH 7 as performed in this project, by titrating NaOH (Fig. 18). The speed and amount of titrated NaOH is then recorded to visualize the *in vitro* lipolysis kinetics of different oral formulations (McClements and Li 2010).

Chapter 2. Main findings, conclusions and future outlook

In this chapter, the concluding remarks of the thesis are presented. The main findings, highlights, and conclusions of each appended scientific paper are summarized. Furthermore, possibilities and needs for further research are suggested.

1. Paper 1: Long-term storage stability of type A and type B gelatin gels: The effect of Bloom strength and co-solutes



This article investigates the long-term storage stability of different gelatin gels. The main results of this study can be summarized in two groups: i) the effect of co-solutes (SAs and gum arabic) on the stability of type B gelatin gels, and ii) the impact of gelatin source (bovine, fish skin or pig skin), pretreatment method (type A or type B), and Bloom strength on the stability of pure gelatin gels. The results were obtained with two main methods: i) SAOS (providing G'_{max} and T_t), and ii) size-Exclusion chromatography coupled to online multi-angle light scattering (SEC-MALS) providing M_w, M_n, and the

rate constant of degradation (k)) for the gels stored at ambient temperature, 30 °C, and 40 °C up to 32 weeks. The pH of all gels were kept constant at 4.5.

The addition of SAs to 160 Bloom type B bovine gelatin gels resulted in higher storage stability by lowering the water activity (a_w) and providing a higher degree of ordering which may reduce the degree of depolymerization. Slower melting/gelling kinetics were observed for the gels with SAs, compared to the control gels. If a less steep temperature gradient and a longer curing time had been used for the SAOS measurements, the gelatin molecules could have had more time to realign, and higher G' could have been recorded for the SA gels. In the future, SAs may be added to type A gelatin gels and the results may be compared to the type B gelatin gel system with SAs. In addition, high and low Bloom strength gelatin gels, along with control gels without any SAs, can be tested to investigate if the stability provided with the inclusion of SAs is more significant than the Bloom strength of gelatin.

The inclusion of gum arabic to 160 Bloom type B bovine gelatin gels did not result in higher storage stability long-term. However, the addition of the gum might have provided a higher degree of order short-term due to weak attractive electrostatic interaction between gum arabic and gelatin. Gum arabic is negatively charged above pH 2 and it is probably not able to form significant attractive electrostatic interactions with type B gelatin at pH 4.5 since this pH value is close to type B gelatin's IEP. However, gum arabic may form attractive electrostatic interactions with type A gelatin at pH 4.5 since type A gelatin would be predominantly positively charged at this pH. As a follow up study, type A and type B gelatin gels should be prepared with gum arabic at different pH values (e.g., pH 3, 4, 5, and 6) and ionic strengths, and the stability of the gels should be investigated with SAOS measurements. Technical issues arose from the complex interactions between gum arabic and gelatin which prevented SEC-MALS analyses on the gelatin gel with gum arabic. If these issues could be resolved, the depolymerization of the gelatin in these systems should also be studied with SEC-MALS.

The results of the study indicated that Type A pig skin gelatin provided gels with better storage stability than type A fish skin gelatin, which had higher storage stability than type B bovine gelatin. Although the paper investigated the depolymerization trends and the bulk properties of the gels, molecular mechanisms of gelatin hydrolysis can be studied with differential scanning calorimetry (DSC), circular dichroism (CD) or optical rotation in a follow-up study. These methods will provide insight on the evolution of the helical content of gelatin during storage and this could shed light on the helical content of the gelatin in the gels over time.

Especially for the type A fish skin gelatin gels, the results obtained with SAOS measurements indicated higher degrees of degradation compared to the results obtained with SEC-MALLS for the gelatin in these systems. In the future, fish gelatin gel samples should be analyzed with a lower curing temperature during the SAOS measurements since the T_m of the fish gelatin gels were very close to the curing temperature of 20 °C, especially towards the end of the storage period.

In addition to the main results obtained from this paper, the follow-up work recommended here can provide a bigger picture for the choice of gelatin source, pretreatment

type, Bloom strength, co-solute type, and pH/ionic strength of the system to be optimized for gelatin-based formulations to obtain high storage stability with pharmaceutical, nutraceutical, and confectionery products.

2. Paper 2: The impact of sugar alcohols and sucrose on the physical properties, long-term storage stability, and processability of fish gelatin gels

This study aims at improving the storage stability of warm water fish gelatin gels with the inclusion of co-solutes: SAs (sorbitol (SO) and xylitol (XY)) or sucrose (SU). Similar to paper 1, the stability of the gels was analyzed after long-term storage at ambient temperature, 30 °C, and 40 °C via SAOS measurements of the gels, and SEC-MALS analyses on the gelatin. In addition, the viscosity of the gelatin solutions was evaluated to assess their processability in the industry. The results of this study can be grouped in three: i) the effect of different co-solutes, ii) the effect of SA concentration, and iii) viscosity of the systems. The pH of the systems were kept constant at pH 4.5.

The a_w and T_t of the gels with co-solutes were lower and higher than the control gel (CO, 0% SA), respectively. However, as the change in the T_t of the gels at all storage temperatures was compared, the ΔT_t of the different co-solute gels (52% SO, XY, and SU) was not significantly different. Similarly, comparing the change in the G'_{max} ($\Delta G'_{max}$) of the gels at all storage temperatures, only CO was significantly different than the co-solute gels. It should be noted that no transition temperature could be obtained for CO after storage at 40 °C for 16 weeks. This prevented the inclusion of week 16 data points for all the gels in statistical tests. The current curing temperature of 20 °C during the SAOS measurements may be too close to the T_g of CO. This issue could be solved by using a lower curing temperature, e.g., 10 °C, for all gels. The change in the M_w (ΔM_w) of the gelatin in the systems after 8 weeks of storage at 30 °C and 40 °C showed significant differences only for CO. In addition, CD measurements can be used to investigate the depolymerization of gelatin in the systems. To conclude, no statistically significant differences were obtained for the effect of different type of co-solutes on the stability of the fish gelatin gels. Browning was observed with SU at 40 °C storage, and this may impact the color and flavor profile of the gel.

The T_t of the gels increased as the SA concentration (CO, M1 (52%), and M2(66%)) increased at all storage temperatures. The ΔT_t of M2 was significantly different than CO. Although only the $\Delta G'_{max}$ of CO was significantly different than the SA gels, the ΔM_w of the gels were significantly different. The results indicated improved storage stability of the fish gelatin gels as the SA concentration increased. Turbidity was observed as M2 was gelled at ambient temperature. This may indicate gelatin aggregation at this concentration of SAs. However, X-ray scattering and CD should be used in the future to understand the molecular mechanisms behind this observation.

The viscosity of gelatin solutions with different co-solutes was compared. SU had the highest viscosity between 50 - 70 °C. This may cause processing challenges for industrial applications. In order to process fish gelatin gels with sucrose, a higher processing temperature should be used, which may compromise the stability of the active ingredients embedded in a potential product. Slower gelling/melting kinetics were observed as the SA concentration increased. The current temperature gradient used for the SAOS mea-

surements may be too rapid for the gelatin molecules to align as the viscosity increased with SA concentration. A lower temperature gradient and a longer curing period should be tested in the future.

Lowering the a_w with the addition of co-solutes resulted in fish gelatin gels which had a higher T_t and better storage stability, making the warm water fish gelatin gels closer in their physical properties to the mammalian gelatin gels. The results indicated that SAs may be a better option than sucrose since they did not cause browning and gave a high viscosity of the samples.

3. Paper 3: Physical and functional properties of plant-based pre-emulsified chewable gels for the oral delivery of nutraceuticals

This study compares simplified versions of agar- and pectin-based CCx to a traditional gelatin CCx. The agar CCx contains citrem and the pectin CCx contains hydrolyzed sunflower lecithin as emulsifier. The emulsion gels were characterized and evaluated regarding their i) a_w, syneresis, gel strength, T_m, and T_g, ii) textural properties, and iii) *in vitro* lipolysis parameters such as total extent of lipolysis and initial lipolysis rate.

The agar and pectin CCx exhibited mild syneresis, whereas the gelatin CCx did not. Mild syneresis may be beneficial to prevent adhesiveness of the gels to the aluminum packaging material. Agar CCx had a T_m of 90 °C, whereas the pectin CCx (53 °C) had a more comparable T_m value to the gelatin CCx (45 °C). Although the high T_m of the agar CCx may be convenient for product transportation and storage in warm climates, the pectin CCx may be a more manufacturable option since it has similar T_m and T_g values to gelatin. On the other hand, the pectin formulation may pose challenges in industrial applications due to its complexity and sensitivity to pH and calcium concentration. The G'_{max} of the CCx was highest for agar, followed by gelatin and with pectin having the lowest.

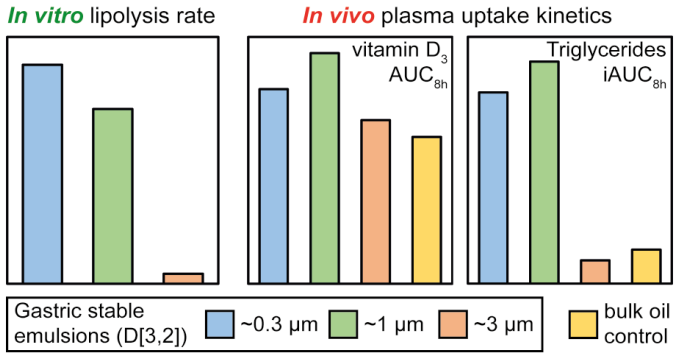
The textural properties of the emulsion gels were evaluated using single compression and texture profile analysis (TPA) at three different maximum strains. The agar CCx had a harder, firmer, and more brittle texture than a gelatin CCx. The pectin CCx was less brittle and softer than the agar CCx. The unique ductile and chewy texture of a gelatin CCx could not be mimicked with any of the polysaccharide gelling agents. However, the pectin CCx exhibited more similar textural properties to the gelatin formulation compared to the agar CCx.

The average droplet size of the polysaccharide emulsion gels were larger than the gelatin CCx, with agar (13 µm) having a smaller droplet size than the pectin CCx (32 µm). The total extent and initial rate of *in vitro* lipolysis was lower for the polysaccharide emulsion gels. For agar, this was most likely due to the lowered emulsification capacity of citrem at gastric pH, resulting in the coalescence of the oil droplets. In addition, the agar matrix will not melt in body temperature, keeping the oil droplets encapsulated. Although the photomicrographs of the pectin CCx after the gastric stage indicated aggregated oil droplets, it had a higher initial lipolysis rate (although not significantly different) and total extent of lipolysis compared to the agar system. The lipolysis curves of the polysaccharide gels did not reach a plateau as the gelatin system, indicating a longer time should be used for the *in vitro* lipolysis of the agar and pectin systems which would also better reflect physiologically relevant digestion time.

One of the biggest product groups in the CCx portfolio is fish oil products. With a potential vegan CCx formulation, fish oil will be replaced by algae oil which is rich in long chain omega-3 fatty acids. The agar and pectin CCx in this study were formulated using corn oil. In a future study, algae oil containing agar and pectin CCx should be

prepared and subjected to stability and oxidation tests. To follow up the *in vitro* lipolysis experiments, *in vivo* experiments and eventually clinical trials should be performed to compare the bioavailability of the CCx systems with different matrix generators. A potential *in vivo* study can follow a similar design as presented in paper 4.

4. Paper 4: The impact of emulsion droplet size on *in vitro* lipolysis rate and *in vivo* plasma uptake kinetics of triglycerides and vitamin D_3 in rats



In this study, the impact of droplet size on the *in vitro* lipolysis and *in vivo* uptake kinetics of emulsions were investigated. Three different droplet size emulsions (small, medium, and large) were prepared using PS80 as the emulsifier. The lipolysis of these emulsions was tested *in vitro*. Three emulsions with these three different droplet sizes were prepared with vitD_3 and E included in the oil phase and their absorption kinetics were studied *in vivo* in rats. The rats were separated in four groups and each group was fed a single dose of different droplet size corn oil emulsion or a bulk oil control. Blood samples were collected from rats and their plasma was analyzed for the lipid-soluble marker molecules, vitD_3 and E, and TAGs. The main results of this article can be summarized in two groups: i) *in vitro* lipolysis, and ii) *in vivo* plasma uptake kinetics.

In vitro, the initial lipolysis rate and the total extent of lipolysis of the emulsions increased as the droplet size decreased. However, the medium droplet size emulsion had significantly higher lipolysis rate per surface area than the small and large droplet size emulsions. The lipolysis rate of the small emulsion may be limited by bile salts to an extent, whereas for the large emulsion the TAG digestion product inhibition could have had an impact. In addition to the main results and discussions on static system limitations, emulsion behaviors in SIF pre-enzyme addition and the optimization of enzyme amounts are presented.

For the *in vivo* experiments with rats, the plasma levels of TAGs, vitD_3 and 25-Hydroxyvitamin D_3 (25-OH vitD_3), i.e., the circulating form of vitD_3, were analyzed using mass spectrometry. TAG levels at $T = 2$, i.e., 2 hours after the administration of the emulsions, was significantly higher for the small and medium emulsions than for the bulk oil control. In addition, the C_{max} and AUC_{8h} for the small and medium emulsions combined were significantly higher than the large emulsion and the bulk oil combined. For vitD_3, the two smallest emulsions, especially the medium emulsion, gave the highest C_{max} and AUC values. However, the differences between the feeding groups in plasma concentrations were not statistically significant. The 25-OH vitD_3 plasma levels increased

from 4 to 24 hour time point for all feeding groups. This indicated saturation of the hepatic conversion process of $vitD_3$ to 25-OH $vitD_3$. Although animal experiments are costly and laborsome, if the period of the sample collection could be expanded, e.g., to 14 days, C_{max} could be observed. Overall, no statistically significant differences in 25-OH $vitD_3$ plasma levels between different feeds were observed. Hemolysis during blood collection from the rats strongly affected the apparent recovery of vitamin E in the emulsions, resulting in the omission of the data from this vitamin. In addition, the co-administration of vitamin E with $vitD_3$ could have reduced the $vitD_3$ uptake due to the saturation of the cholesterol membrane transporters, resulting in active uptake pathway competition between the two vitamins.

In a future study, gelled gelatin-based emulsions, i.e., a simplified gelatin CCx, can be first tested *in vitro*. Then gelled emulsions can be prepared with $vitD_3$ alone and be fed to the rats in a single large dose. The gels can be split into small pieces to mimic mastication and be fed using a suitable oral gavage. Three different droplet size emulsions can be obtained with different homogenization times. The uptake kinetics can be compared to a bulk oil control with a marker molecule, e.g., $vitD_3$. Plasma samples can be collected and the levels of $vitD_3$ can be analyzed using mass spectrometry. Rats have a different physiology than humans. Therefore, it would be ideal to test the gelled emulsions in clinical trials. It would also be of interest to test alternative fat soluble actives, in which the commercial impact of increased bioavailability is important.

5. Paper 5: A comparative study of different plant proteins as potential emulsifiers in agar containing emulsions

In this study, six different plant proteins (pea, soy, rice, potato, faba bean, and chickpea protein) were tested as emulsifiers in a simplified agar CCx. The emulsifying properties of the plant proteins were first investigated without agar or SAs in the formulation by preparing: i) ambient emulsions (at native pH 6.5 - 7.5, without any buffer salts), and ii) formulation emulsions (at pH 4.4 - 4.6, with malic acid (MA) and trisodium citrate dihydrate (TCD)). The ambient emulsions had better emulsifying properties than formulation emulsions. At ambient pH pea protein stabilized emulsion had the smallest $D[4,3]$ droplet size average and highest emulsion activity index (EAI). At formulation relevant pH, the faba bean stabilized emulsion had the smallest $D[4,3]$ average, whereas chickpea stabilized emulsion had the highest EAI. Pea protein stabilized emulsion also had the highest emulsion stability index (ESI) at both pH values. The impact of ionic strength on the emulsifying properties of pea protein were studied by comparing the pea protein stabilized formulation emulsion (ionic strength = 0.3 M) with an NaCl emulsion (ionic strength = 0.15 M). The emulsion showed better emulsifying properties at ionic strength 0.3 M in the presence of buffer salts.

Agar CCxs were prepared using different plant proteins as emulsifiers. In this paper, the stability of the CCxs were studied before they were gelled, i.e., while they were still in a liquid state. Therefore, the CCxs were referred to as agar containing liquid emulsions (ACLEs). ACLEs were kept in liquid state for 24 hours (t_{24}) after their preparation (t_0). ACLEs prepared with pea, rice, and potato protein showed a minor degree of creaming and/or coalescence at t_{24}. The droplet size averages and distributions indicated that the ACLEs did not show drastic destabilization at t_{24} and pea protein stabilized emulsion had the smallest change in droplet size. The thermal loop method was used to investigate the heat stability of the ACLEs. Potato, faba bean, and chickpea protein stabilized ACLEs showed structural changes at 70 °C which may be due to their partial denaturation at this temperature.

The results indicated that the current formulation pH of 4.5 may be too close to the isoelectric point of the proteins. Considering all the tests, pea, faba bean, and chickpea protein showed promising emulsifying properties in the agar system and these proteins should be further tested in an agar formulation with a lower pH value. This study was performed as an initial screening of the plant proteins in the agar system. In the future, replicate emulsions and emulsion gels should be tested with the utilized methods and the emulsifying properties of the plant proteins should be compared using statistical methods. In addition, the solubility, dispersibility, and hydrophobicity of the proteins should be evaluated in a follow up study.

6. Concluding remarks of the thesis

With this work, it has been proven possible to improve the storage stability of gelatin-based gels with the correct choice of gelatin type, Bloom strength and the inclusion of sugar alcohols (**Paper 1**). It was shown that the stability of warm water fish gelatin gels can also be improved with the addition of sugar alcohols without compromising the processability (**Paper 2**). It has been proven that plant-based polysaccharides can be used to replace gelatin as matrix generators, although the physical and functional properties of the emulsion gels prepared with these polysaccharides will be different compared to gelatin-based ones (**Paper 3**). It was also shown that plant-proteins have potential as emulsifiers in agar emulsion gels (**Paper 5**). This work has also shown that smaller oil droplets will be lipolyzed faster than larger oil droplets *in vitro*. However, this was not reflected to the *in vivo* uptake of the lipid-soluble active ingredients but was only valid for the TAGs (**Paper 4**). The findings in this thesis can be used to improve the stability, physical, and functional properties of chewable emulsion gels for the oral delivery of nutraceuticals.

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Appended papers and patent application

Paper 1. Long-term storage stability of type A and type B gelatin gels: The effect of Bloom strength and co-solutes

Baydin, T., O. A. Aarstad, M. J. Dille, M. N. Hattrem, and K. I. Draget (2021). "Long-term storage stability of type A and type B gelatin gels: The effect of Bloom strength and co-solutes". In: *Food Hydrocolloids* 127, pp. 107535. DOI:10.1016/j.foodhyd.2022.107535.

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Long-term storage stability of type A and type B gelatin gels: The effect of Bloom strength and co-solutes

Tuna Baydin [a,b,*], Olav A. Aarstad [a], Morten J. Dille [a,b], Magnus N. Hattrem [b], Kurt I. Draget [a,b]

[a] Department of Biotechnology and Food Science, Norwegian University of Science and Technology, 7491, Trondheim, Norway
[b] Vitux AS, Brynsveien 11, 0667, Oslo, Norway

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ABSTRACT

For gelatin-based confectionery, nutraceutical, and pharmaceutical products, storage stability is important for maintaining textural properties during transportation and throughout the shelf life of the final product. Exposure to elevated temperatures causes degradation of the gelatin molecule, which results in a reduction in the average molecular weight, further impacting its mechanical properties. In this study, the long-term stability of gelatin systems prepared with different gelatins (type B from bovine, type A from pig skin, or type A from fish skin) stored below and above the sol-gel transition temperature was compared. The effect of storage temperature, Bloom strength, and the inclusion of co-solutes (sugar alcohols or gum arabic) on storage stability was evaluated. The long-term stability was investigated using small amplitude oscillatory shear measurements and molecular weight analyses via size-exclusion chromatography coupled with online multiangle laser light scattering. The storage modulus of the gels and the average molecular weight of the gelatin indicated that incubation above or close to the equilibrium sol-gel transition temperature resulted in an increased degradation rate of gelatin. Type A gelatin gels exhibited better storage stability than type B gelatin gels. In addition, gels prepared with high Bloom strength gelatins exhibited improved storage stability compared to low Bloom strength gelatins. The addition of sugar alcohols increased stability, whereas gum arabic did not have a large impact on the long-term stability of the type B gelatin gel.

1. Introduction

Gelatin is a common ingredient in a wide range of products, including foods, pharmaceuticals, and nutraceuticals (Schrieber & Gareis, Practical Aspects, 2007). It forms thermo-reversible gels with an equilibrium sol-gel transition temperature (T_t) close to the physiological temperature of the animal the gelatin is derived from (Djabourov & Papon, 1983). Thus, mammalian gelatin has a T_t close to human body temperature (Joly-Duhamel, Hellio, & Djabourov, 2002). This property gives the gel a characteristic texture with a melt-in-the-mouth perception (Dille & Draget, 2020; Dille, Haug, & Draget, 2021; Haug & Draget, 2011). Due to these properties, gelatin is commonly used as an ingredient in confectionery jellies, as well as in different types of delivery systems for pharmaceuticals and nutraceuticals, such as soft gel capsules, hard gel capsules and chewables. Although the gel has solid-like properties when it is set, after ingestion, the gelatin-based product will be exposed to body temperature, causing it to rapidly dissolve and release its active ingredient.

Gelatin-based products, including gummies, may be exposed to elevated temperatures during storage and transportation. In warm climates, the storage temperature may exceed 40 °C for certain periods. Textural stability influences how the mouth-feel, texture, consistency, and shelf life of the product change over time (International Food Information Council (IFIC) and U.S. Food and Drug Administration (FDA), 2018). The storage stability of gelatin is an integral factor for the industry since degradation occurs at elevated temperatures (Ames, 1947). In this study, storage stability stands for the ability of the gel to maintain its mechanical properties such as toughness, strength, and elasticity at a specific temperature over time (International Union of Pure and Applied Chemistry, 2008).

The U.S. Food and Drug Administration (FDA) recommends a low pH value (below 4.6) in food products as a preventative measure against microbial growth (2018). Lowering the pH value of the product also presents some challenges, as it promotes acid hydrolysis, causing cleavage of peptide bonds during storage, further impacting the textural stability (Koli, Basu, Kannuchamy, & Gudipati, 2013; van den Bosch &

* Corresponding author. Department of Biotechnology and Food Science, Norwegian University of Science and Technology, 7491, Trondheim, Norway.
E-mail address: tuna.baydin@ntnu.no (T. Baydin).

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Gielens, 2003). Storage temperature is also important in terms of the rate of acid hydrolysis. Long exposure to temperatures above the T_t results in accelerated degradation of the gelatin because of the acid hydrolysis of peptide bonds in the unordered coil of the gelatin molecule. Degradation may also occur below the T_t (Schrieber & Gareis, Practical Aspects, 2007; van den Bosch & Gielens, 2003).

The FDA also recommends reducing the water activity below 0.85 in confectionery products in order to prevent microbial growth (2018). Reducing water activity is also often beneficial for the chemical stability of food products, improving textural stability and shelf life (Fontana, 2000). In this context, sugar alcohols such as the polyols xylitol, sorbitol, mannitol, erythritol, and glycerol, are widely used as alternatives to sugars and glucose syrup. In addition, polyols provide sweetness, texture, and body to the final product (Poppe, 1992). One benefit of using sugar alcohols is the low calorie content of these compounds. In addition, they contribute to dental health and offer a non-sugar alternative for people with diabetes (Tau & Gunasekaran, 2016).

Gum arabic, commonly used as a food ingredient, obtained from the exudate of the *Acacia senegal* tree, is a complex polysaccharide which contains a small amount of nitrogenous material (Anderson, Hendrie, & Munro, 1972; Williams & Phillips, 2009). In gelatin-based products, gum arabic is used as an emulsifier, formulation aid, stabilizer, and thickener (U.S. Food and Drug Administration, 2020). Gum arabic is negatively charged above pH 2 due to the presence of carboxylic acid groups (Burgess & Carless, 1984; Gulão, de Souza, da Silva, Coimbra, & Garcia-Rojas, 2014). It has been reported that above pH 2, some local patches in the proteinaceous content of gum arabic carry positive charges (Sabet et al., 2021). Gum arabic and gelatin form attractive electrostatic interactions in the range of pH 3.5–4.3 where gelatin may undergo complex coacervation with gum arabic (Anvari, Pan, Yoon, & Chung, 2015; Bungenberg de Jong & Kruyt, 1929; Burgess & Carless, 1984; Evans, Ratcliffe, & Williams, 2013; Peters, van Bommel, & Fokkens, 1992; Yang, Anvari, Pan, & Chung, 2012).

Type A and type B gelatin are produced through the partial hydrolysis of collagen by acid or alkali, respectively (Eastone & Leach, 1977). Generally, type A gelatin is obtained from pigs, poultry, and fish, whereas type B gelatin is obtained from bovine sources. Type B gelatin contains approximately 65% more aspartic acid and glutamic acid residues than type A gelatin because of the deamidation of asparagine and glutamine under alkaline conditions, resulting in an increased number of negative charges (Rose, 1987). Consequently, type B and type A gelatin have an isoelectric point (IEP) at pH 5 and 8.5, respectively (Schrieber & Gareis, From Collagen to Gelatine, 2007). In general, fish gelatin contains a lower amount of imino acids, i.e., hydroxyproline and proline, and a higher amount of serine and threonine, compared to mammalian gelatins (Balian & Bowes, 1977). Therefore, gelatins from cold water fish exhibit lower gelling-melting point and gel strength compared to mammalian gelatin (Derkach, Voron'ko, Kuchina, & Kolotova, 2020). However, fish gelatin from warm water species exhibits physical and rheological properties similar to those of mammalian gelatins (Gilsenan & Ross-Murphy, 2000).

With confectionery and gummies, a low pH value is typically used to ensure microbial stability and to provide sourness, which yields an optimal final product if combined with organic acids, aromas, sweeteners, and colorants. Due to the sensitivity of gelatin gels to acid hydrolysis, a pH value of 4.5 compromises microbial stability, good taste, and long-term textural stability. In this study, the pH value was kept at 4.5 for the different gelatin gels, using malic acid and trisodium citrate dihydrate. Citric acid, malic acid, and their salts are among the most commonly used acidulants for confectionery, and they synergistically provide more sourness when combined in a formulation (Sortwell, 2014).

The impact of several parameters on the storage stability of gelatin gels as well as the molecular, physical, and rheological characteristics of gels made with gelatin from different sources have been previously investigated (Eysturskard, Haug, Ulset, & Draget, 2009; Eysturskard, Haug, Ulset, Joensen, & Draget, 2010; Hattrem, Molnes, & Draget, 2014; Haug, Draget, & Smidsrød, 2004; Leuenberger, 1991). Previous experiments on the heat degradation of gelatin were conducted at temperatures ranging from 40 to 100 °C. However, the heating time did not exceed 13 days (Ames, 1947; Qi et al., 2018; van den Bosch & Gielens, 2003). To the authors' knowledge, a comprehensive and comparative long-term stability study with realistic storage conditions extending to 32 weeks, including gelatin gels with and without co-solutes, has not been done. The scope of this work was to compare the impact of the gelatin source (type A from pig skin or fish skin and type B from bovine) and Bloom strength on the long-term storage stability of gelatin gels at a constant pH of 4.5. In addition, sugar alcohols or gum arabic were combined with type B gelatin gels to analyze their impact on storage stability. It was hypothesized that type B gelatin gels may be less stable than type A gelatin gels because of the higher negative charge density of the former, promoting proton condensation and acid hydrolysis. Moreover, the inclusion of sugar alcohols was expected to increase the stability of gelatin gels by decreasing the water activity and providing a more ordered gelatin conformation. The addition of gum arabic to gelatin gels was expected to increase gel stability through attractive electrostatic interactions with gelatin.

2. Materials and methods

2.1. Materials

Bovine and porcine skin gelatin (Bovine 260 Bloom Batch: 642516, Bovine 160 Bloom Batch: 643208, Pig skin 260 Bloom Batch: 643060, Pig skin 160 Bloom Batch: 643190) were obtained from Gelita®, Germany. Progel warm water fish gelatin from *Pangasius hypophtalmus* skin was obtained from Vinh Hoan Corporation, Vietnam. Gum arabic (type 8013, 0800193) was purchased from HPI GmbH, Germany. Xylitol was provided by Danisco, UK. Sorbitol was purchased from Food Innovation, Norway. Malic acid and trisodium citrate dihydrate were obtained from MerckMillipore, USA.

2.2. Composition, preparation and storage of gelatin gels

Gelatin gels were prepared using gelatin from different sources (bovine, pig skin, or fish skin) and Bloom strengths (160 g, 200 g, or 260 g). Trisodium citrate dihydrate and malic acid were used as buffer salts to maintain a stable pH of approximately 4.5. Gels containing 160 Bloom bovine gelatin were prepared with or without sugar alcohols (xylitol and sorbitol) or gum arabic with the compositions listed in Table 1. The gelatin/water ratio was kept constant among all gels, except for the gel with gum arabic.

25 (w/w)% gelatin was mixed with deionized water (18.2 MΩcm Stakpure OmniaPure, Germany) containing 0.02 (w/w)% sodium azide (NaN_3, BDH, UK) for 30 min with magnetic stirring. The temperature throughout the preparation of the gel solution was maintained at 60 °C. 2 (w/w)% trisodium citrate dihydrate was added and mixed for 10 min. 1 (w/w)% malic acid was added and mixed for another 10 min. The concentrations of trisodium citrate dihydrate and malic acid were optimized to obtain a pH value close to 4.5. 0.05 (w/w)% Witafrol was added to prevent foaming, and the mixture was degassed twice with a diaphragm vacuum pump (Vacuubrand, MZ 2C, Germany). Any water loss was readded after degassing. The pH was measured using a pH combination electrode (A 162 2M DI, SI Analytics, Germany) connected to a titrator (7000-M1/20, TitroLine, SI Analytics, Germany), and 5 M HCl was manually added until a stable pH of 4.5 was reached. 4 (w/w)% gum arabic powder was added with the gelatin. 23 (w/w)% sorbitol and 35.2 (w/w)% xylitol were added after 30 min of mixing 10 (w/w)% gelatin with water. After the addition of the sugar alcohols, the mixture was magnetically stirred for 10 min before the addition of trisodium citrate dihydrate. Following the pH stabilization step, the gels were poured into glass containers, and the lid was tightly covered with

Table 1
The abbreviations and compositions of gelatin gels with different gelatin types, Bloom strengths and co-solutes. All ingredients are given as percentage (w/w).

Gelatin type	Bloom strength (g)	Abbreviation	Gelatin (%)	Gum arabic (%)	Sugar alcohols (%)	Buffer salts (%)	Water (%)
Type B – bovine	260	260B	25	–	–		72
	160	160B	25	–	–		72
	160	160B-GA	25	4	–	Trisodium citrate dihydrate 2	68
	160	160B-SA	10	–	Sorbitol 23 Xylitol 35.2		28.8
Type A – pig skin	260	260A	25	–	–	Malic acid 1	72
	160	160A	25	–	–		72
Type A – fish skin	200	200A-Fish	25	–	–		72

parafilm to prevent evaporation during storage. After sealing, the containers were immediately placed in 30 °C and 40 °C temperature-controlled chambers and at ambient temperature, i.e., an average of 22 °C laboratory environment. These temperatures were chosen to represent subtropical, tropical, and temperate zones, respectively.

2.3. Rheology

Rheological analyses of the gels were performed using a rheometer (Malvern Kinexus ultra+, Westborough, United States) using a CP4/40 40 mm diameter 4° angle cone and a 50 mm diameter sandblasted lower plate with curved edges. The day of gel preparation was considered to be t_0. At selected time points (weeks 1, 2, 4, 8, 16 and 32), approximately 2 g of gel sample was removed from storage, placed on the pre-heated lower plate (60 °C) and allowed to melt. The rheometer was operated in 0.1% shear strain-controlled mode, and the frequency was set to 1 Hz. The chosen strain was confirmed to be within the linear viscoelastic region for all samples. To avoid evaporation, the gel sample was covered with silicone oil (10 cS fluid, Dow Corning, UK) prior to and during measurement. The viscoelastic properties of the sample were obtained using a temperature gradient of 2 °C/min, with a starting and an ending temperature of 60 °C and holding time of 15 min at 20 °C. The results were analyzed using the rSpace for Kinexus software. The maximum storage modulus (G') (Pa) was determined to be the highest measurement point during curing at 20 °C. Maximum measured storage modulus (G'_{max}) at t_0 was considered as baseline, and the differences in G'_{max} at later time points (weeks 1, 2, 4, 8, 16 and 32) were subtracted from the t_0 value to obtain the change in G'_{max}, i.e., $\Delta G'_{max}$. The differences were normalized to calculate the percent change. The gelling temperature (T_g and melting temperature (T_m) of the samples were estimated as the temperature at which the phase angle corresponding to 45° (i.e., $\delta = 45°$) in the cooling and heating processes, respectively. T_t was calculated as the numerical average of T_m and T_g.

2.4. SEC-MALLS

The molecular weight distributions of the gelatin in the gels were analyzed by size-exclusion chromatography coupled with online multi-angle laser light scattering (SEC-MALLS). At each analysis time point, 200 mg of gel was frozen in an Eppendorf tube at −30 °C for further analysis. At selected time points, 100 mg of gel was placed into a new Eppendorf tube and diluted 5X with elution buffer (0.1 M Na2SO4, 0.02 M EDTA, pH value adjusted to 9). The mixture was heated to 60 °C and further diluted with elution buffer to obtain a gelatin concentration of 2 mg/ml in each sample. The mixture was filtered with a syringe filter (0.45 μm, Millex-HP, Cork, Ireland) and stored in an HPLC vial (VWR, 1.5 ml, Darmstadt, Germany) at −30 °C prior to analysis. To represent t_0 sample for each gel, a fresh gelatin solution with the corresponding gelatin type was prepared. The t_0 solutions were treated prior to SEC-MALLS analysis in the same way as described above for the gelatin gels.

SEC-MALLS was performed at 40 °C on an HPLC system consisting of a solvent reservoir, online degasser, column oven from Waters, Agilent 1260 Infinity II pump, and an automatic sample injector. The samples were separated on a serially connected TSK gel pre-column and PWXL 6000 + 5000 main columns (Tosoh Bioscience, Japan). The column outlet was connected to a DAWN HELEOS-II MALLS photometer (Wyatt Technology, USA) ($\lambda_0 = 663.8$ nm) and a Shodex RI-501 refractive index detector. The eluent was 0.1 M Na_2SO_4 with 0.02 M EDTA (pH 9), delivered at a flow rate of 0.5 ml/min. Each sample was analyzed with injection volumes of 25, 50, and 100 μl. Pullulan (137 kDa) was included as the standard, and dextran (11.2 kDa) was used for normalization. Data were collected and processed (with dn/dc = 0.190 ml/g for gelatin) using Astra software (version 7.3.0, Wyatt Technology, USA).

Statistical analyses were performed using IBM SPSS Statistics version 27. Equalities of variances were analyzed using Levene's test. One-way analysis of variance with post-hoc Tukey's honest significant difference test was performed for multiple comparisons. Statistically significant differences in the $\overline{M_n}$ and $\overline{M_w}$ of gelatin in different gels were reported if $P < 0.05$.

The rate constant (k) was calculated using Eq. (1), where M_0 is the average molar mass of an amino acid (~100 Da), and $\overline{M_n}$ is the number average molecular weight at a time point.

$$\frac{1}{\overline{M_n}} = \frac{1}{\overline{M_{n,0}}} + \frac{k \cdot t}{M_0} \quad (1)$$

t represents time, and indicates the time points at t_0, week 4, and week 16. The k values at 30 and 40 °C were normalized to the k value at ambient temperature and are given as $k_{relative}$.

3. Results

3.1. Rheology

3.1.1. Effect of the presence of co-solutes

Sugar alcohols are known to affect the gelling-melting behavior and mechanical properties of gelatin gels (Gekko, Xuan, & Makino, 1992; Ling, 1978; Miyawaki, Omote, & Matsuhira, 2015; Oakenfull & Scott, 1986; Shimizu & Matubayasi, 2014). Therefore, the impact of sugar alcohols on the storage stability of the gelatin gels was investigated. The changes in the $\Delta G'_{max}$ and T_t of type B – 160 Bloom gelatin gel with (160B-SA) and without (160B) polyols were compared.

At t_0, 160B (25 (w/w)% gelatin) had a higher G'_{max} compared to 160B-SA (10 (w/w)% gelatin), with 17.9 kPa and 8.0 kPa, respectively. After 32 weeks of storage, 160B-SA had a smaller decrease in G'_{max} compared to 160B at all three storage temperatures. 160B-SA retained significant elastic character, i.e., $G' >> 0$, even after 16 weeks of storage at 40 °C (Fig. 1).

Although the initial T_t of 160B-SA was much higher than 160B, at ambient temperature, the T_t of both gels showed a similar decrease after 32 weeks. The apparent thermal hysteresis also increased with the inclusion of sugar alcohols at all storage temperatures (Supplementary Figs. 1A and 1B). Throughout storage at 30 °C, as the T_t of 160B decreased from above 30 °C to below 30 °C, the T_t of 160B-SA was above 30 °C for 32 weeks. At 40 °C, the T_t of 160B-SA after 32 weeks was still higher than the T_t of 160B after 8 weeks (Table 2).

The impact of gum arabic on the storage stability of gelatin gels was investigated by comparing type B – 160 Bloom gelatin gel with (160B-

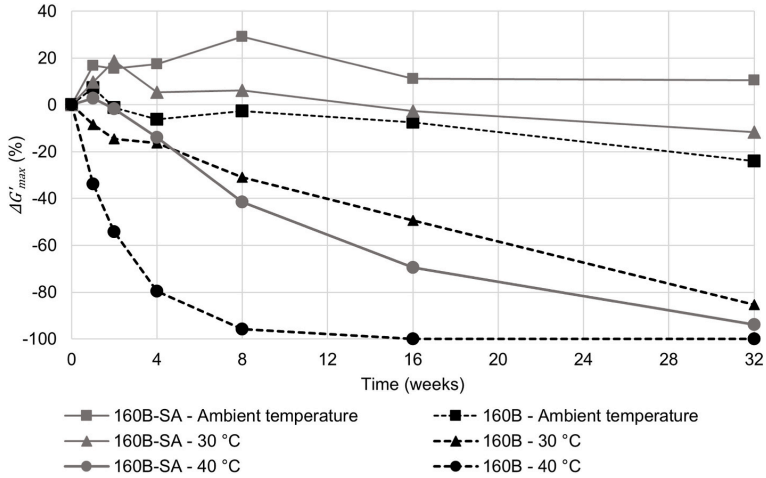

Fig. 1. The change in G'_{max} of type B – 160 Bloom gelatin gel with (gray, 160B-SA) and without (black, 160B) sugar alcohols (23 (w/w)% sorbitol and 35.2 (w/w)% xylitol) over 32 weeks of storage. The $\Delta G'_{max}$ is given as percent change compared to its t_0 value. Different storage temperatures are shown as: (■) ambient temperature, (▲) 30 °C, and (●) 40 °C. The data points are connected to guide the eye.

Table 2
The equilibrium sol-gel transition temperature (T_t) °C for different gels stored at ambient temperature (A), 30 °C, or 40 °C. The abbreviations used for each gel are given in Table 1. N/A (not available) indicates that no transition temperature could be determined as the solution did not solidify under the current gelling regime and temperature gradient.

Weeks	260B			160B			160B-GA			160B-SA		
	A	30 °C	40 °C	A	30 °C	40 °C	A	30 °C	40 °C	A	30 °C	40 °C
0 (t_0)	31.9	31.9	31.9	30.5	30.5	30.5	31.8	31.8	31.8	39.6	39.6	39.6
1	32.4	31.6	29.5	30.6	29.9	27.6	31.5	30.9	28.6	39.7	39.0	37.7
2	31.9	31.2	27.8	30.1	29.7	26.1	31.7	30.6	27.6	39.7	39.2	37.0
4	31.7	30.3	25.8	29.9	28.8	24.1	31.7	29.9	25.1	39.4	38.3	34.5
8	31.2	30.3	23.1	29.7	28.1	22.3	30.8	29.3	23.1	39.5	37.7	32.4
16	30.4	28.2	N/A	29.1	27.0	N/A	30.6	27.9	N/A	38.5	36.2	28.4
32	29.4	26.8	N/A	28.3	24.8	N/A	29.2	26.2	N/A	37.7	34.7	24.0

Weeks	260A			160A			200A-Fish		
	A	30 °C	40 °C	A	30 °C	40 °C	A	30 °C	40 °C
0 (t_0)	33.4	33.4	33.4	30.1	30.1	30.1	25.0	25.0	25.0
1	34.3	33.3	31.1	30.4	30.2	28.6	25.2	24.8	24.0
2	33.5	33.1	29.5	30.5	29.9	27.6	25.2	24.6	23.6
4	33.5	32.6	27.8	30.4	29.6	26.0	24.9	24.2	22.7
8	33.0	31.2	25.4	30.0	28.9	24.3	24.9	23.5	21.4
16	32.2	30.3	22.5	29.9	28.4	21.1	24.8	22.7	N/A
32	31.0	28.5	N/A	28.9	26.4	N/A	24.4	20.8	N/A

GA) and without (160B) gum arabic, as described above. At t_0, 160B-GA (25 (w/w)% gelatin) had a slightly higher G'_{max} compared to 160B (25 (w/w)% gelatin), with 18.5 kPa and 17.9 kPa, respectively. At ambient temperature, 160B-GA exhibited an increase in G'_{max} after storage for 16 weeks, whereas the control gel without gum arabic showed a decrease in G'_{max} starting from week 2. At the end of 32 weeks, both gels showed similar reductions in G'_{max} upon storage at ambient temperature, 30 °C, and 40 °C (Fig. 2).

At t_0, the T_t of 160B-GA was slightly higher than for 160B. This initial increase is consistent with the literature (Binsi et al., 2017). At ambient temperature, 30 °C, and 40 °C, the decrease in the T_t of both gels over time followed a similar tendency with a slightly smaller decrease for 160B-GA (Table 2, Supplementary Figs. 2A and 2B).

3.1.2. Effect of gelatin type and Bloom strength

Based on consumer preference and desired rheological properties of the final product, different raw material sources (bovine, porcine, poultry, fish, etc.) and pretreatment methods (acid or alkaline) are used in the production of gelatin. The impact of the gelatin source (bovine, pig skin, or fish skin), pretreatment method (type A or type B), and Bloom strength on the storage stability of pure gelatin gels were analyzed. In this study, "pure gel" is used to describe an aqueous gelatin gel in the presence of buffer salts, but without any other co-solutes (sugar alcohols or gum arabic). Gels stored at 30 °C were selected to show the change in rheological characteristics (G'_{max} and T_t) of pure gelatin gels using small amplitude oscillatory shear (SAOS) measurements. The impact of storage at ambient temperature, 30 °C, and 40 °C on $\Delta G'_{max}$ is given in Supplementary Fig. 3, Supplementary Fig. 4, and Supplementary Fig. 5, respectively.

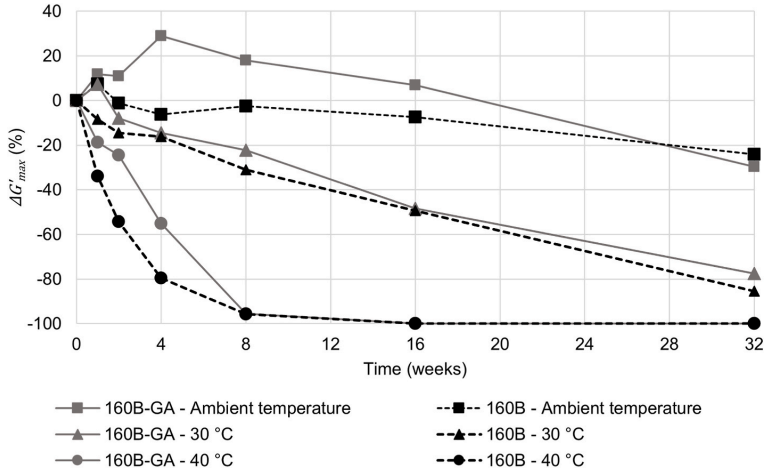

Fig. 2. The change in G'_{max} of type B – 160 Bloom gelatin gel with (gray, 160B-GA) and without (black, 160B) gum arabic (4 (w/w)%) over time. The $\Delta G'_{max}$ is given as percent change compared to its t_0 value. Different storage temperatures are shown as: (■) ambient temperature, (▲) 30 °C, and (●) 40 °C. The data points are connected to guide the eye.

After 4 weeks of storage at 30 °C, type A pig skin gelatin gels showed an increase in G'_{max}, in contrast to type B gelatin gels. After 32 weeks of storage at 30 °C, all pure gelatin gels exhibited lower G'_{max} values with variances in the decrease between gels. The decrease in G'_{max} at the end of 32 weeks of storage at 30 °C was: 200A-Fish > 160B > 260B > 160A > 260A (Fig. 3).

After storage at 30 °C for 32 weeks, the T_t of type B gelatin gels and type A pig skin gelatin gels decreased from above 30 °C to below 30 °C. The T_t of 200A-Fish was below 30 °C throughout the whole storage period. For the type B bovine and type A pig skin gelatin gels, the gels made with the higher Bloom strength gelatin had a higher T_t at t_0 and at week 32. At 32 weeks, the T_t values of the pure gelatin gels were: 260A > 260B > 160A > 160B > 200A-Fish (Table 2).

3.2. SEC-MALLS

3.2.1. Effect of sugar alcohols

To investigate the correlation between the changes in rheological properties, weight average molecular weight ($\overline{M_w}$), and $\overline{M_n}$, SEC-MALLS analyses were performed on gelatin from 160B and 160B-SA at selected time points, i.e., t_0, week 4, and week 16, after storage at ambient temperature, 30 °C, or 40 °C.

Both 160B and 160B-SA showed a decrease in the $\overline{M_w}$ and $\overline{M_n}$ of gelatin over the study period. This decrease was larger at longer storage times, higher temperatures and without sugar alcohols (Fig. 4). The raw data of all gelatin gels analyzed using SEC-MALLS in this study are shown in Supplementary Table 1.

An alternative way to show the change in the average molecular weight of the gelatin is the cumulative weight fraction (w), which is the weight fraction of molecules in the sample with a molecular weight less

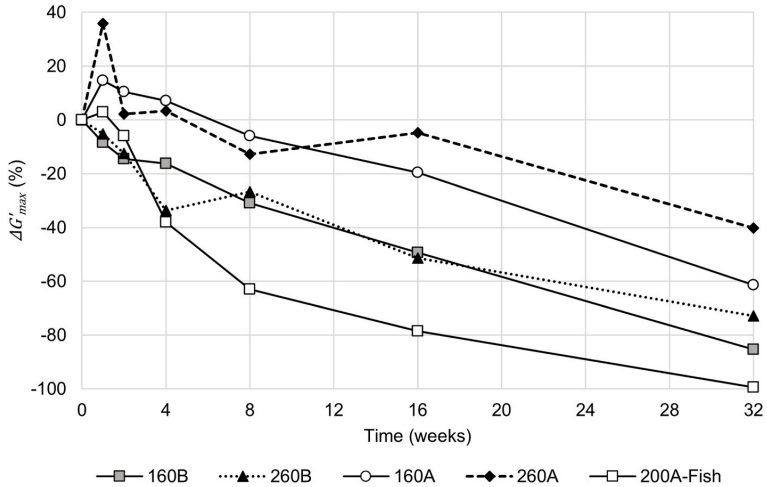

Fig. 3. The change in G'_{max} of gelatin gels from three different sources over 32 weeks storage at 30 °C. Two different Bloom strength type B and type A – pig skin gelatins are shown. The $\Delta G'_{max}$ is given as percent change compared to its t_0 value. The data points are connected to guide the eye.

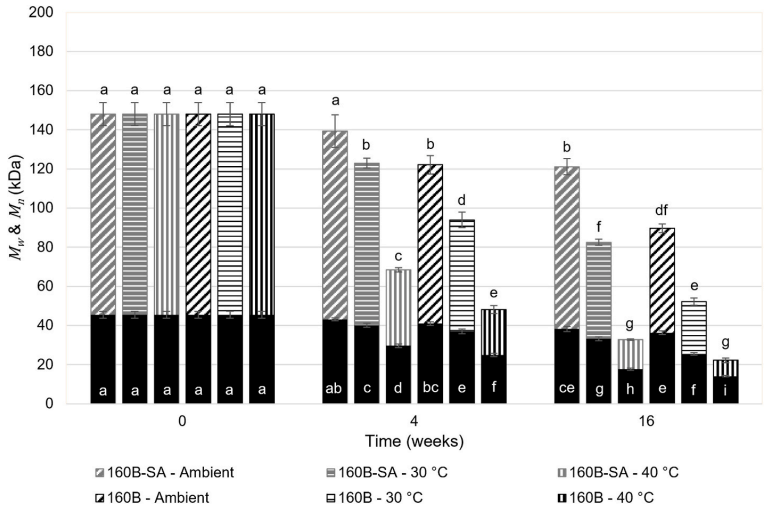

Fig. 4. $\overline{M_w}$ and $\overline{M_n}$ measured using SEC-MALLS for 160B-SA and 160B upon storage at ambient temperature, 30 °C, and 40 °C up to 16 weeks. Black bars show $\overline{M_n}$, and patterned bars show $\overline{M_w}$. The results are given as $\overline{M_w}$ or $\overline{M_n}$ (kDa) ± standard deviation, n = 3 injections were performed for each sample. Different letters above the patterned column, and inside the black columns indicate statistically significant differences ($P < 0.05$) for $\overline{M_w}$ and $\overline{M_n}$, respectively.

than M (Roger & Colonna, 1996; Shortt, 1933). The gelatin molecular weight M at cumulative weight fractions, $W = 0.5$ and $W = 0.9$ ($M_{W=0.5}$ *and* $M_{W=0.9}$) at t_0 and after 16 weeks of storage at different temperatures are shown in Supplementary Table 2.

As a standard estimation of the hydrolysis rate, the rate constant (k) was calculated using Eq. (1). k provided a numerical value that enabled the comparison of the hydrolysis rate of a gel at different storage temperatures or different gels at a particular storage temperature.

160B-SA exhibited smaller k values compared to the control gelatin gel without sugar alcohols at all storage temperatures. However, $k_{relative}$ increased approximately three times and twice with a 10 °C increase from ambient temperature to 30 °C, for 160B and 160B-SA, respectively (Table 3).

3.2.2. *Effect of gelatin type and Bloom strength*

Similar to the gelatin gels with sugar alcohols, SEC-MALLS analyses were performed on pure gelatin gels stored at 30 °C. The reduction in $\overline{M_w}$ and $\overline{M_n}$ was larger for type B gelatin than type A gelatin. For both type A and type B gelatins, the k value was lower for the high Bloom (260 g) gelatin compared to the low Bloom (160 g) gelatin (Fig. 5).

The $M_{W=0.5}$ *and* $M_{W=0.9}$ for different gelatin gels at t_0 and after 16 weeks of storage at 30 °C can be found in Supplementary Table 3.

The k values indicated the rate of hydrolysis for the pure gelatin gels at 30 °C in the following order: 160B > 260B > 200A-Fish >160A > 260A (Table 4).

Table 3
k values of 160B and 160B-SA at different storage temperatures.

Gel	Temperature (°C)	k ($weeks^{-1}$)	$k_{relative}$
160B	Ambient	$3.3 \cdot 10^{-5}$	1.0
	30 °C	$10.6 \cdot 10^{-5}$	3.3
	40 °C	$29.4 \cdot 10^{-5}$	9.0
160B-SA	Ambient	$2.6 \cdot 10^{-5}$	1.0
	30 °C	$4.8 \cdot 10^{-5}$	1.9
	40 °C	$20.9 \cdot 10^{-5}$	8.2

4. Discussion

Generally, the inclusion of sugar alcohols is known to increase the gel strength of gelatin gels (Gekko et al., 1992; Oakenfull & Scott, 1986). At t_0, the higher G'_{max} of 160B compared to 160B-SA may be explained by the lower gelatin per volume in 160B-SA. It could also, at least in part, be due to slower kinetics in the formation of ordered structures in the presence of polyols, considering the relatively short curing time at 20 °C. The T_t represents the transition temperature from disordered coils to ordered single helices, and is calculated as the numerical average of the T_m and T_g (Bohidar & Jena, 1993; Miyawaki et al., 2015; Parker & Povey, 2012). For gelatin gels, the numerical difference between the measured values of T_m and T_g, i.e., apparent thermal hysteresis, is primarily due to the non-instant sol-gel transitions during the applied temperature gradient (Djabourov & Papon, 1983; Djabourov, Leblond, & Pappon, 1988). It should be noted that the T_m and T_g values calculated in this study were obtained with a temperature gradient of 2 °C/min. The transition temperatures are expected to vary between different temperature gradients. The T_t of the type B gelatin gel increased with the inclusion of sugar alcohols, indicating that the gels retained their solid-like behavior at higher temperatures (Table 2).

The gelling and melting curves for 160B-SA were less steep than those for 160B, indicating slower gelling and melting kinetics upon sugar alcohol addition (Supplementary Fig. 6). The observed increases in the T_m and T_g are consistent with the literature (Eysturskard, 2010; Gekko & Koga, 1983; Joly-Duhamel et al., 2002). The majority of the gelatin molecules will be in a disordered state, i.e., the sol fraction will dominate, at temperatures exceeding the T_t of the gelatin gel (Bohidar & Jena, 1993). The disordered coils are more prone to degradation by hydrolysis since they are more accessible to the solvent (van den Bosch & Gielens, 2003). For other biopolymers, such as xanthan, it is known that molecules in an ordered conformation are less susceptible to degradation and therefore are more thermally resistant than molecules in a disordered conformation (Lund, Lecourtier, & Müller, 1990). At 30 °C, the majority of the gelatin molecules in the 160B-SA gels were in an ordered state, as opposed to the gels without sugar alcohols. However, at 40 °C, the majority of the gelatin molecules in both gels are expected to be in the disordered random coil state and therefore should be more prone to hydrolysis. The relative drop in T_t was lower for 160B-SA than

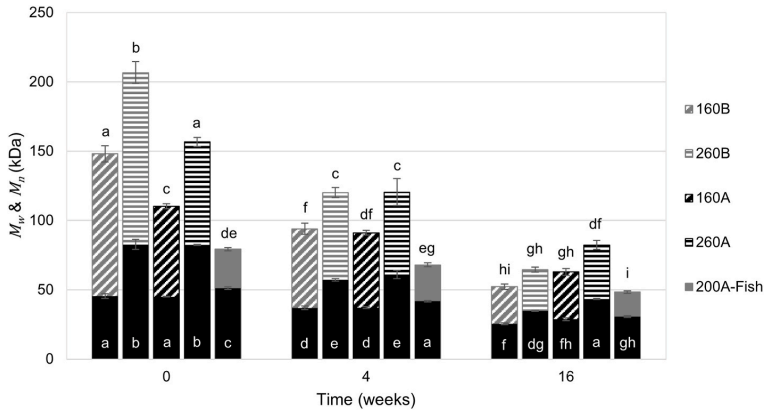

Fig. 5. $\overline{M_w}$ and $\overline{M_n}$ measured using SEC-MALLS for different pure gelatin gels upon storage at 30 °C up to 16 weeks. Black bars show $\overline{M_n}$, and patterned bars show $\overline{M_w}$. The results are given as $\overline{M_w}$ or $\overline{M_n}$ (kDa) ± standard deviation, n = 3 injections were performed for each sample. Different letters above the patterned column, and inside the black columns indicate statistically significant differences ($P < 0.05$) for $\overline{M_w}$ and $\overline{M_n}$, respectively.

for 160B, at all storage temperatures. At this temperature, the T_t of 160B-SA after 32 weeks was still higher than the T_t of 160B after 8 weeks (Table 2). Furthermore, 160B-SA had a k value 29% lower than 160B at 40 °C, indicating a slower hydrolysis rate, possibly due to the relatively higher degree of ordering with the inclusion of sugar alcohols (Table 3). In this study, linear trendlines of $1/\overline{M_n}$ versus t resulted in $R^2 > 0.95$ for each gel, confirming that there was one rate constant throughout the storage of the gels (data not shown). Although the k value is estimated for the hydrolysis of one linkage, different bonds may be hydrolyzed at similar rates.

There are different theories for the stabilization mechanisms of gelatin using sugar alcohols. Miyawaki et al. suggest that reduced water activity due to the coexistence of solutes, such as polyols, in solution increases the intra and intermolecular hydrogen bonding and hydrophobic interactions among protein molecules by changing solvent ordering (Miyawaki et al., 2015). Shimizu and Matubayasi suggest that the exclusion of sugar alcohols from the surface of gelatin molecules promotes gelation by driving protein-protein interactions (Shimizu and Matubayasi, 2014). Gekko et al. argue that sugar alcohols stabilize gelatin due to a large decrease in the entropy gain of gel melting (Gekko et al., 1992). Oakenfull and Scott argues that polyols stabilize gelatin gels by increasing the thermodynamic stability of the collagen fold. As a result, the number of collagen triple helix junction zones increases, which contributes to a more extended gel network (Oakenfull and Scott, 1986). All of these studies indicate that sugar alcohols increase the degree of ordering within gelatin gels, which may reduce the degree of depolymerization. Current data support previous studies suggesting increased stability of gelatin with the addition of sugar alcohols, indicating that sugar alcohols contribute to the storage stability of gelatin gels by lowering the water activity and providing a higher degree of ordering of the gelatin molecule (Fig. 4) (Gekko & Koga, 1983; Gekko et al., 1992). This influence became clearer as the storage temperature increased from ambient temperature to 40 °C (Table 3).

In previous studies, the presence of gum arabic significantly reinforced the gelatin network due to attractive electrostatic interactions between gum arabic and fish gelatin, resulting in a more compact network structure of the gelatin gel (Anvari et al., 2015; Binsi et al., 2017). In the current study, no major differences were found between 160B and 160B-GA, indicating that the current pH value of 4.5 may be too high for gum arabic to form significant attractive electrostatic interactions with type B gelatin. Furthermore, the strength of any electrostatic interaction will be reduced with the increased ionic strength in the present system (Vinayahan, Williams, & Phillips, 2010). On the other hand, one could still expect attractive electrostatic interactions between gum arabic and type A gelatin since at pH 4.5 gum arabic would be negatively charged, and type A gelatin would be predominantly positively charged. Therefore, the type of gelatin may have an impact on the electrostatic interactions with gum arabic due to different charge distributions and IEPs (Burgess & Carless, 1984; Peters et al., 1992). The impact of gum arabic on the storage stability of gelatin gels from different sources, i.e., type A and type B, at different pH values should be investigated further.

The results of the storage modulus and the gelling-melting kinetics of 160B and 160B-GA suggest that gum arabic may induce a lag phase, which provides a higher degree of short-term order due to weak attractive electrostatic interactions between gum arabic and gelatin (Fig. 2, Supplementary Figs. 2A and 2B). However, gum arabic does not contribute to the long-term storage stability of gelatin gels. The changes in $\overline{M_w}$ and $\overline{M_n}$ of 160B-GA were not investigated using SEC-MALLS in this study. This was due to complex interactions between gum arabic and gelatin, which influenced the reliability of the analysis.

For all gelatin gels, an increase in storage temperature led to a significantly faster decline in G'_{max} during storage, as expected (Supplementary Fig. 3, Supplementary Fig. 4, and Supplementary Fig. 5). Considering the change in T_t, one week of storage of 160B at 40 °C roughly equaled 32 weeks of storage under ambient conditions

Table 4
k values of pure gelatin gels upon storage at 30 °C up to 16 weeks.

Gel	k (weeks^{-1})
160B	$10.6 \cdot 10^{-5}$
260B	$10.1 \cdot 10^{-5}$
160A	$7.6 \cdot 10^{-5}$
260A	$6.5 \cdot 10^{-5}$
200A-Fish	$8.0 \cdot 10^{-5}$

(Table 2). This may suggest a 30-fold increase in the rate of hydrolysis caused by an increase in the storage temperature of approximately 20 °C. A similar trend was observed for the same gel with the $\Delta G'_{max}$ results (Fig. 1). However, the k values indicate a 9-fold increase comparing the hydrolysis rates at ambient temperature and 40 °C (Table 3). It should be noted that unlike SEC-MALLS measurements, the SAOS experiments do not directly reflect the hydrolysis rate of gelatin in the gels. There appeared to be no linear relationship between the hydrolysis rate of gelatin and the reduction in the T_t or $\Delta G'_{max}$ of the gelatin gels. For the SAOS measurements, the gels were reheated to 60 °C, and different peptide linkage susceptibility to acid hydrolysis when the gel was in the gelled versus sol state may be of significance. Van den Bosch and Gielens state that polypeptide splittings occur next to N-terminal glycine, hydroxyproline, proline and alanine residues in gelatin gels incubated at 40 °C, suggesting that these residues may be the most susceptible to hydrolysis (van den Bosch & Gielens, 2003). The triple helical junction zones, i.e., Pro-Hyp-Gly, are crucial for the gelling properties of gelatin since they help stabilize the α-chain (Haug et al., 2004; Ross-Murphy, 1992). The triple helical junction zones may be protected by the ordered state, as also observed with ordered xanthan (Lund et al., 1990).

The rheological characterizations indicated that type A pig skin gelatin gels may have better storage stability than type B gelatin and fish gelatin gels. The increase in the G'_{max} of type A pig skin gelatin gels up to 4 weeks of storage at 30 °C, unlike type B gelatin gels, may be due to the formation of hydrogen bonds between asparagine residues in type A gelatin and the peptide backbone (Fig. 3) (Vijayakumar, Qian, & Zhou, 1999). Throughout storage at 30 °C, for both type B and pig skin type A gelatin gels, the higher Bloom strength (260 g) gelatin gel exhibited less reduction in G'_{max} compared to the low Bloom strength (160 g) gelatin gel (Fig. 3). The Bloom value and $\overline{M_w}$ are proportional, and higher values result in a lower sol and loose end fraction, forming a more optimally structured gel with longer triple helices, and a higher number of intermolecular bonds that are more resistant to hydrolysis (Hattrem et al., 2014; Netter, Goudoulas, & Germann, 2020). The k value was smaller for gelatin gels containing high Bloom gelatins, confirming that the results obtained with the SAOS measurements were most likely due to acid hydrolysis, for both type A and type B gelatin (Table 3). As in accordance with the literature, for both pig skin type A and type B gelatin gels, 260 Bloom gelatin gels had a higher T_t, compared to 160 Bloom gelatin gels at t_0 (Table 2) (Choi & Regenstein, 2000; Gilsenan & Ross-Murphy, 2000; Hattrem et al., 2014; Netter et al., 2020). In addition, type A pig skin gelatin gels showed a smaller reduction in T_t compared to the type B gelatin gels upon storage at 30 °C for 32 weeks. Similar results were observed for storage at ambient temperature and at 40 °C (Table 2). At 30 °C storage, type B gelatin showed a larger decrease in $\overline{M_w}$ and a higher k value compared to type A gelatin (Fig. 5 and Table 4). Because of the alkaline pretreatment of type B gelatin, there are 46 aspartic acid and 72 glutamic acid residues, whereas there are only 29 aspartic acid and 48 glutamic acid residues (per 1000 residues) in type A gelatin (Schrieber & Gareis, From Collagen to Gelatine, 2007). Consequently, proton condensation around the negative charges explains why type B gelatin would be more susceptible to acid hydrolysis than type A gelatin, since increasing proton density increases the rate of intramolecular acid hydrolysis (Eigen, 1964; O'Connor, 1970; Rose, 1987; Schrieber & Gareis, From Collagen to Gelatine, 2007). This effect was observed for many anionic polymers. For example, negatively charged sulfate groups in κ-carrageenan locally attract protons, which causes an increase in the rate of acid hydrolysis (Hjerde, Smidsrød, & Christensen, 1996).

A lower T_t value for fish skin gelatin compared to the mammalian gelatins was expected because of its lower content of imino acids (Choi & Regenstein, 2000; Gilsenan & Ross-Murphy, 2000; Veis, 1964). At 30 °C, 200A-Fish was assumed to have an increased sol-fraction due to its lower T_t, which was below 30 °C throughout the 32 weeks of storage. Although 200A-Fish showed the largest decrease in G'_{max} compared to mammalian gelatins at this temperature, the average SAOS results suggested a dominant solid-like behavior up to 16 weeks (Fig. 3). Concurrently, the results of the SEC-MALLS analyses indicated that fish gelatin exhibited the lowest reduction in $M_{W=0.5}$ *and* $M_{W=0.9}$ compared to the other gelatins after 16 weeks (Supplementary Table 3). 200A-Fish showed a hydrolysis rate faster than that of the pig skin type A gelatins, but slower than that of the type B gelatins (Table 4). It should be noted that 200A-Fish is a type A gelatin with less proton condensation, which may explain why it is more resistant to hydrolysis than type B gelatins. At a glance, the SAOS and SEC-MALLS measurements on 200A-Fish pose contradictory results, which however can be explained by the measurement conditions used for the SAOS measurements. The curing of the gel occurred at 20 °C, and the G'_{max} was measured at this temperature, which is very close to the T_g of 200A-Fish; the curing time was also relatively short. Therefore, a small decrease in T_g results in a large decrease in the G'_{max} since it hardly gels at 20 °C. This limitation might have influenced the results obtained with the current setup, and a lower curing temperature should be tested for 200A-Fish.

5. Conclusions

In this study, the long-term storage stability of type A and type B gelatin gels with different Bloom strengths was investigated. For type B gelatin gels, the impact of adding co-solutes, i.e., sugar alcohols and gum arabic, was also examined. As expected, an increased rate of gelatin hydrolysis was observed with increasing storage temperature. For both type A and type B gelatin, the high Bloom (260 g) gelatin gels exhibited a lower rate of hydrolysis compared to the low Bloom (160 g) gelatin. Overall, type A gelatin gels exhibited a lower rate of hydrolysis compared to type B gelatin gels throughout long-term storage, possibly due to the lower negative charge density of type A gelatin. The inclusion of sugar alcohols provided a higher transition temperature of the resulting gel and also resulted in a lower degree of hydrolysis, most likely through an increased degree of ordering within the gelatin gels. Complementary results indicated that the hydrolysis of gelatin can be traced and, to a certain extent, quantified by quick and relatively simple SAOS measurements instead of more tedious and complex SEC-MALLS analyses. The current findings might contribute to the choice of gelatin raw material source, type, Bloom strength and inclusion of sugar alcohols for gelatin-based products. Especially in warm climates, a high Bloom strength type A gelatin and/or addition of sugar alcohols may be beneficial in formulations to achieve high storage stability of gelatin-based confectionery, nutraceuticals, and pharmaceuticals.

Author statement

Tuna Baydin: Conceptualization, Methodology, Data curation, Visualization, Writing - original draft, Formal analysis, Validation.

Olav A. Aarstad: Investigation, Methodology, Data curation, Software.

Morten J. Dille: Formal analysis, Writing - review & editing.

Magnus N. Hattrem: Conceptualization, Supervision, Writing - review & editing.

Kurt I. Draget: Resources, Project administration, Conceptualization, Supervision, Writing - review & editing.

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Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2022.107535.

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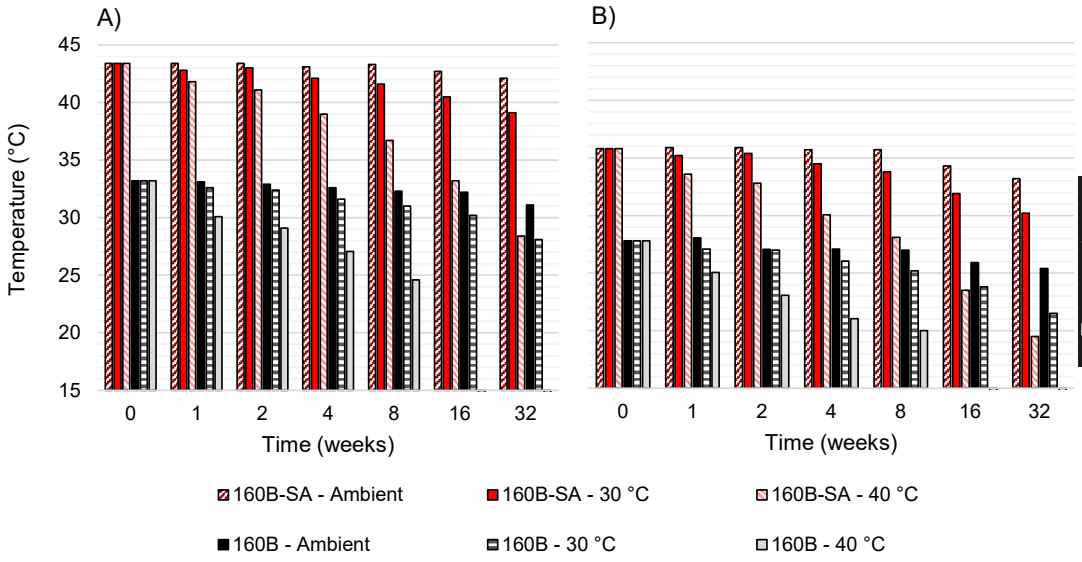
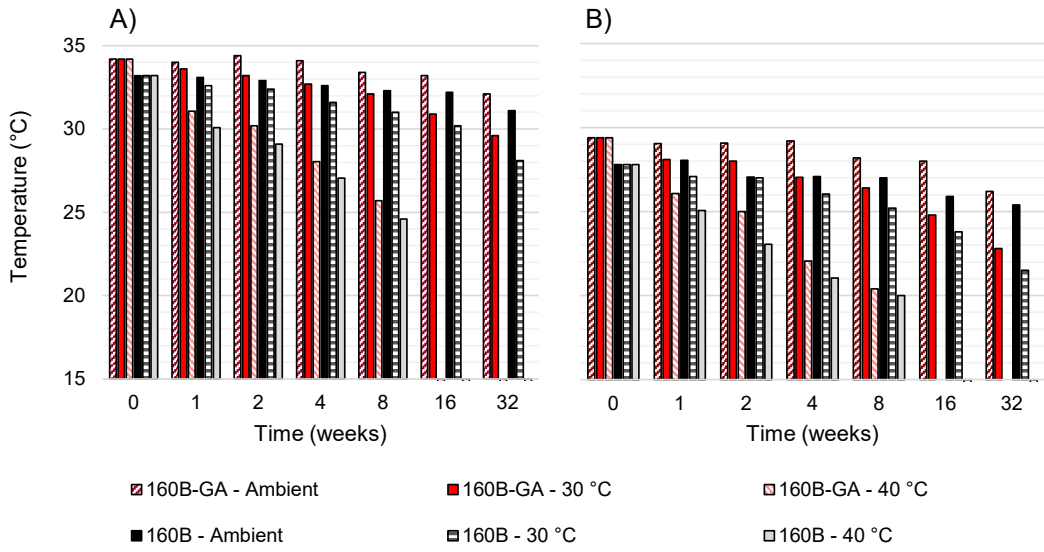
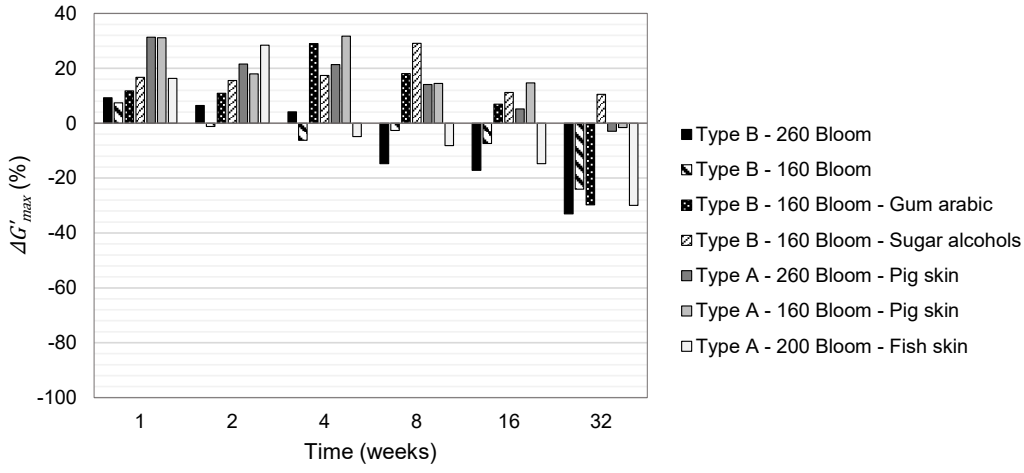
Supplementary Data



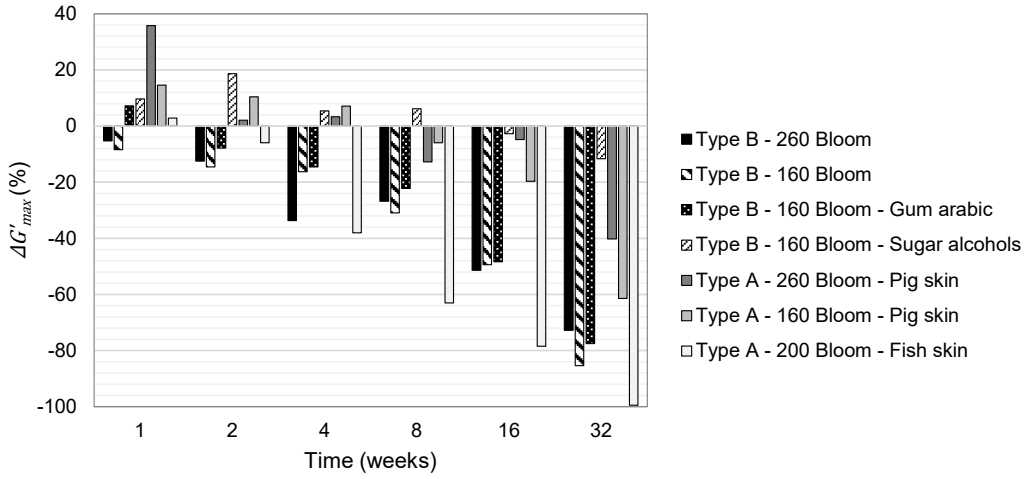
Supplementary Figure 1: A) Melting temperature (T_m) and B) gelling temperature (T_g) of type B – 160 Bloom gelatin gel with (160B-SA) and without (160B) sugar alcohols over 32 weeks storage at ambient temperature, 30 °C or 40 °C. Empty bars at week 16 and 32 indicate too low G' values to detect a temperature corresponding to 45° phase angle.



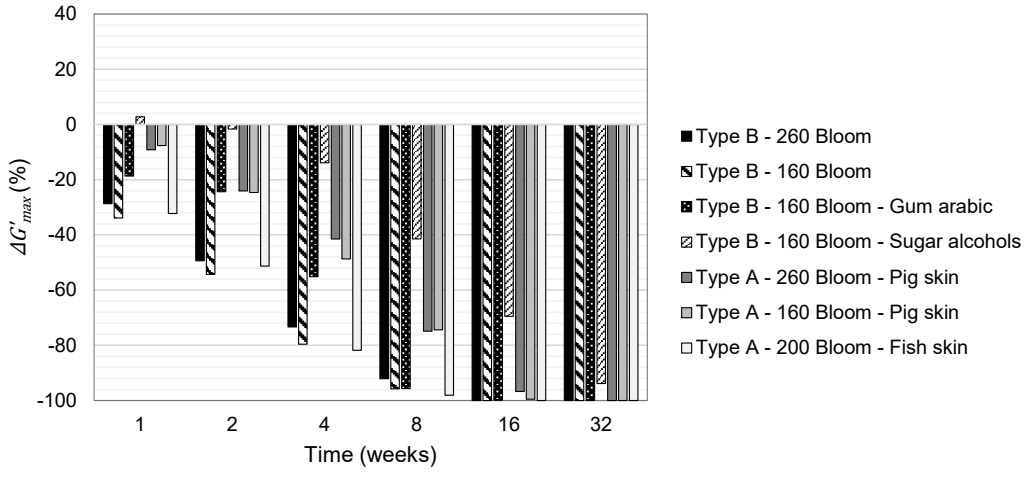
Supplementary Figure 2: A) Melting temperature (T_m) and B) gelling temperature (T_g) of type B – 160 Bloom gelatin gel with (160B-GA) and without (160B) gum arabic over 32 weeks storage at ambient temperature, 30 °C or 40 °C. Empty bars at week 16 and 32 indicate too low G' values to detect a temperature corresponding to 45° phase angle.



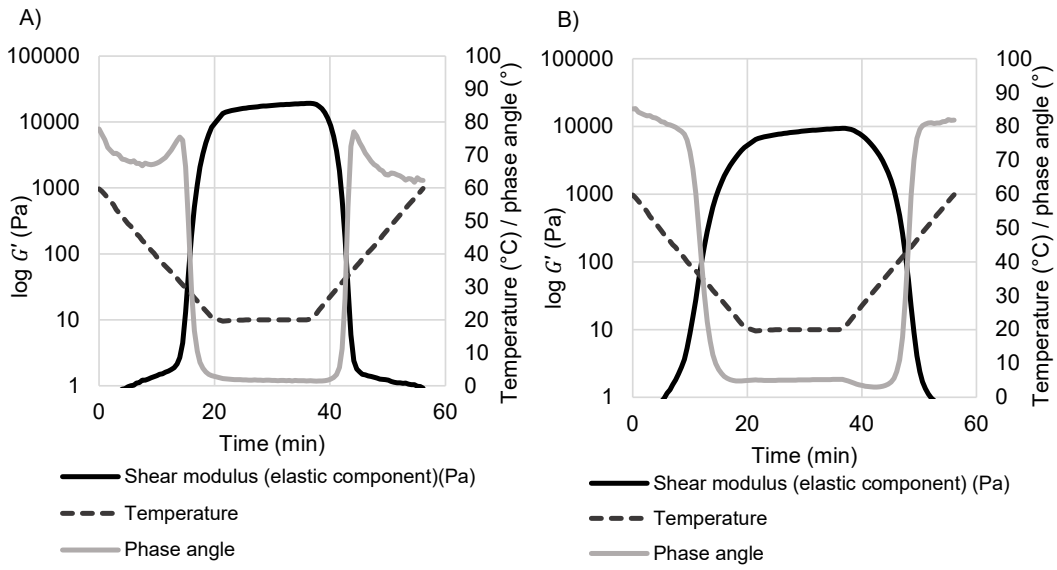
Supplementary Figure 3: The change in G'_{max} after storage of different gelatin gels at ambient temperature for 32 weeks. The $\Delta G'_{max}$ is given as percent change compared its t_0 value for each gel.



Supplementary Figure 4: The change in G'_{max} after storage at 30 °C for 32 weeks. The $\Delta G'_{max}$ is given as percent change compared its t_0 value for each gel.



Supplementary Figure 5: The change in G'_{max} after storage at 40 °C for 32 weeks. The $\Delta G'_{max}$ is given as percent change compared its t_0 value for each gel.



Supplementary Figure 6: Small amplitude oscillatory shear measurements of A) 160B and B) 160B-SA after one week storage at ambient temperature.

Supplementary Table 1: Raw data obtained with the SEC-MALLS measurements for gelatin gels stored at different temperatures. Three injections were performed for each sample and the injected mass was 116.7 µg.

Gel	Storage temperature	Time point	$\overline{M_n}$ (kDa)	$\overline{M_w}$ (kDa)	Polydispersity ($\overline{M_w}/\overline{M_n}$)	Mass recovery (%)
260B		t_0	82.7	206.7	2.5	73.2
	Ambient	week 4	70.4	158.7	2.3	79.9
		week 16	55.6	113.6	2.1	79.4
	30 °C	week 4	82.7	206.7	2.0	74.3
		week 16	57.1	120.1	1.9	72.6
	40 °C	week 4	34.8	64.5	1.8	76.8
		week 16	15.4	23.7	1.5	73.2
160B		t_0	45.4	148.1	3.3	71.5
	Ambient	week 4	41.0	122.2	3.0	84.9
		week 16	36.3	89.7	2.5	77.0
	30 °C	week 4	37.0	94.0	2.5	76.7
		week 16	25.5	52.2	2.1	77.2
	40 °C	week 4	25.0	48.2	1.9	75.9
		week 16	14.1	22.3	1.6	73.4
160B-SA		t_0	45.4	148.1	3.3	71.5
	Ambient	week 4	43.1	139.4	3.2	76.4
		week 16	38.2	121.1	3.1	75.1
	30 °C	week 4	40.1	123.0	3.1	74.1
		week 16	33.3	82.5	2.5	73.7
	40 °C	week 4	29.8	68.5	2.3	73.4
		week 16	17.7	32.8	1.9	72.5
260A		t_0	82.3	156.7	1.9	69.6
	30 °C	week 4	61.1	120.5	2.0	74.3
		week 16	43.5	82.3	1.9	72.6
160A		t_0	45.0	110.3	2.5	67.0
	30 °C	week 4	37.2	91.1	2.4	67.7
		week 16	28.7	62.9	2.2	67.4
200A-Fish		t_0	51.2	79.2	1.5	72.9
	30 °C	week 4	41.7	68.0	1.6	78.5
		week 16	30.6	48.3	1.6	77.3

Supplementary Table 2: The M of gelatin from 160B and 160B-SA at cumulative weight fraction $W = 0.5$ and $W = 0.9$ at t_0 and 16 weeks of storage at ambient temperature, 30 °C or 40 °C. The values are given as M ± standard deviation (kDa) of 3 replicates with injection volumes of 25, 50, and 100 µl.

		$M_{W=0.5}$ (kDa)	$M_{W=0.9}$ (kDa)
	t_0	80.3 ± 1.6	341.1 ± 11.4
Gel	Storage temperature		
160B	Ambient	53.9 ± 0.8	189.6 ± 6.1
	30 °C	34.8 ± 0.4	103.8 ± 3.6
	40 °C	16.1 ± 0.4	40.4 ± 1.7
160B-SA	Ambient	66.5 ± 1.2	264.7 ± 8.9
	30 °C	49.2 ± 0.6	173.4 ± 3.1
	40 °C	23 ± 0.1	64.9 ± 1.1

Supplementary Table 3: The M of gelatin from the pure gelatin gels at cumulative weight fraction $W = 0.5$ and $W = 0.9$ at t_0 and 16 weeks of storage at 30 °C. The values are given as M ± standard deviation (kDa) of 3 replicates with injection volumes of 25, 50, and 100 µl.

Gel	Time	$M_{W=0.5}$ (kDa)	$M_{W=0.9}$ (kDa)
160B	t_0	80.3 ± 1.6	341.1 ± 11.4
	week 16	34.8 ± 0.4	103.8 ± 3.6
260B	t_0	97.6 ± 1.5	463.4 ± 21.5
	week 16	43.3 ± 0.6	124 ± 3.4
160A	t_0	70.4 ± 0.5	247.4 ± 4.2
	week 16	39.2 ± 1.3	142.1 ± 7.9
260A	t_0	103.8 ± 1.8	338.9 ± 8.7
	week 16	53.1 ± 1.2	182.8 ± 8.2
200A-Fish	t_0	63.8 ± 0.7	153.6 ± 1.9
	week 16	37.1 ± 0.8	92.4 ± 2.4

Paper 1

Paper 2. The impact of sugar alcohols and non-reducing sugars on the physical properties, processability, and long-term stability of fish gelatin gels

Baydin, T., M. J. Dille, O. A. Aarstad, M. N. Hattrem, and K. I. Draget (2023). "The impact of sugar alcohols and sucrose on the physical properties, long-term storage stability, and processability of fish gelatin gels". In *Journal of Food Engineering* 341, pp.111334. DOI: 10.1016/j.jfoodeng.2022.111334.

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The impact of sugar alcohols and sucrose on the physical properties, long-term storage stability, and processability of fish gelatin gels

Tuna Baydin [a,b,*], Morten J. Dille [a,b], Olav A. Aarstad [a], Magnus N. Hattrem [b], Kurt I. Draget [a,b]

[a] Department of Biotechnology and Food Science, Norwegian University of Science and Technology, Trondheim, Norway
[b] Vitux AS, Oslo, Norway

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ABSTRACT

Fish gelatin is becoming a popular alternative to mammalian gelatins due to religious restrictions, cultural preferences, ecological, and ethical concerns. Warm water fish gelatins (WWFG), as opposed to gelatins from cold water fish species, have more similar physical properties, and hence represent an alternative, to mammalian gelatins albeit a lower sol/gel transition temperature and gel strength. In warm climates, WWFG gels may therefore exhibit reduced storage stability at temperatures above this transition temperature because of e.g., a more pronounced acid hydrolysis of the sol fraction. To improve the long-term storage stability of WWFG, gels were prepared with two different sugar alcohols, sorbitol and xylitol, and a non-reducing sugar, sucrose. The change in the sol/gel transition temperature, gelling and melting kinetics, and gel strength of the gels were analyzed using small amplitude oscillatory shear measurements. Short-term and long-term storage stability tests at ambient temperature, 30 °C and 40 °C indicated improved stability of gels with co-solutes without significant differences between the type of sugar alcohol or sucrose. The stability of the gels increased with increasing concentrations of sugar alcohols. The degree of hydrolysis of the gelatin in the gels were investigated using SEC-MALS analyses which supported the bulk rheology stability results. Using sucrose led to browning and high viscosity, which may pose challenges regarding the processability and industrial applications.

PAPER 2

1. Introduction

Gelatin is a versatile ingredient with many functional properties, and it is used in the preparation of foods, pharmaceuticals, and nutraceuticals. It forms thermoreversible gels, yields "melt-in-the-mouth" texture and possesses surface active properties. Gelatin can also be used to enhance the stability, elasticity, chewiness, and consistency of food products (Choi and Regenstein, 2000; Dille et al., 2021; Montero and Gómez-Guillén, 2000; Schrieber and Gareis, 2007). Gelatin is obtained through the partial hydrolysis of collagen from the connective tissues of animals including cattle, pigs, poultry, and fish (Schrieber and Gareis, 2007). It has been reported that 98.5% of gelatin consumed worldwide is obtained from pig skin, cattle hides, and cattle bones, and only 1.5% is obtained from fish-derived sources and poultry (Karim and Bhat, 2009; Milovanovic and Hayes, 2018). In Europe, 80% of edible gelatin is produced from pig skin, 15% is sourced from cattle skin, and the remaining 5% comprises porcine and bovine bones, poultry and fish (Boran and Regenstein, 2010; GME, 2021). The majority of pharmaceutical and food-grade gelatin is hence obtained from mammalian sources. Mammalian gelatins are widely used because their equilibrium sol-gel transition temperature (T_t), i.e., coil-helix transition temperature, is close to the physiological temperature of humans. This gives gelatin gels the familiar melt-in-the-mouth texture, appreciated by consumers of confectionaries and desserts. In addition, it provides gelatin-based delivery forms with consistent and rapid dissolution in the gastrointestinal tract, being an important step in the oral administration of pharmaceuticals and nutraceuticals. However, there are some limitations regarding the usage of mammalian gelatin, including religious and ethical constraints. Islam and Judaism prohibit the consumption of pork and non-religiously slaughtered cow-related food products, and Hinduism prohibits products derived from cattle. The outbreak of Bovine spongiform encephalopathy has also raised concerns about the risk of infection upon consumption of foods derived from cattle (Grobben et al., 2004). In addition, cardiovascular health issues, ecological, ethical, and philosophical considerations have increased the number of individuals following a pescatarian or vegetarian diet (Wozniak et al., 2020). Therefore, the interest and demand for alternatives to meat-based products have increased and this demand is also emerging in

* Corresponding author. Department of Biotechnology and Food Science, Norwegian University of Science and Technology, Trondheim, Norway.
E-mail address: tuna.baydin@ntnu.no (T. Baydin).

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the gelatin industry (Anzani et al., 2020; Karim and Bhat, 2009).

In this context, fish gelatin poses as a potential alternative to mammalian gelatins. No major religious practices prohibit its consumption, and its production has a smaller carbon footprint compared to gelatin production from mammalian sources (Nijdam et al., 2012). The production of fish gelatin relies on byproducts from the fish industry which yields approximately 75% of the total wild catch weight including head, bone, skin, and viscera (Shahidi, 1994; Wasswa et al., 2007). However, the production and consumption of fish gelatin is currently limited. This may be attributed to the suboptimal properties of gelatin obtained from cold water fish which constitute the majority of the wild catch (Leuenberger, 1991). On the other hand, gelatin obtained from warm water fish, which constitutes the majority of farmed fish species, has physical properties much closer to mammalian gelatins (Nations, 2014; Sarabia et al., 2000). This is attributed to the higher content of imino acids (hydroxyproline and proline) in WWFG, which is more similar to the contents found in mammalian gelatin (Haug et al., 2004; Leuenberger, 1991).

When the storage temperature exceeds the T_t of a gelatin gel, the sol fraction will dominate, and the majority of the gelatin molecules will be in a disordered state (Bohidar and Jena, 1993). If the gelatin-based product is exposed to higher temperatures, this may lead to failure in the uniformity of the product, leakage of the encapsulated ingredient (in soft-gel capsules) and more rapid hydrolysis of the gelatin molecule impacting the long-term storage stability of the product (Baydin et al., 2022; Wang and Hartel, 2022a). As WWFG has a lower T_t compared to mammalian gelatin, products prepared with such gelatin are more susceptible to temperature-mediated degradation (Dille et al., 2021). This gives further limitations with regards to temperature control in the supply chain of such products and may potentially lead to quality deviations. Hence, it is of large interest to increase the T_t and improve the storage stability for products prepared with WWFG.

Different approaches have been used to optimize and improve the physical properties and storage stability of WWFG gels. Derkach et al. (2020) proposed adding natural ionic polysaccharides that form polyelectrolyte complexes with gelatin to eliminate disadvantageous characteristics of fish gelatin. For these gels, the pH of the system plays an important role since the complex formation depends on attractive electrostatic interactions between gelatin and the polysaccharide in a particular pH range. Other suggestions to improve the physical properties and stability of fish gelatin gels include the use of enzymatic or chemical cross-linkers, ethanol, and salts (Benjakul et al., 2012; Chiou et al., 2006; Elysée-Collen and Lencki, 1996; Yi et al., 2006). These modifications increase the complexity of the formulation and may not be well-received by the consumers who are inclined to natural and clean eating (Rozin, 2005).

Sugar alcohols, also known as polyols, are obtained by the reduction of an aldo or keto group of a sugar to the corresponding hydroxy group (Bieleski, 1982). The EU legislation identifies seven sugar alcohols: sorbitol, mannitol, isomalt, maltitol, lactitol, xylitol and erythritol (Mortensen, 2006). These bulk sweeteners are commonly used as sugar substitutes in food products since they provide, in addition to sweetness, texture, and body to the final product (Poppe, 1992). Benefits of sugar alcohols include a low calorie content, improved dental health favoring the lack of microbial growth in dental plaque, and being suitable for patients with diabetes since polyols do not induce an increase in blood glucose or insulin, as much as traditional sugars (Grembecka, 2015; Tau and Gunasekaran, 2016). In addition to aforementioned benefits, sugar alcohols contribute to reducing the water activity (a_w) in food systems since they reduce the amount of free water in the system (Miyawaki et al., 2003). The U.S. Food and Drug Administration (2018) recommends a a_w of 0.85 or below to prevent microbial growth in food products. Reducing the a_w also improves the chemical stability, textural stability, and shelf life of food products (Fontana, 2000).

It is also known that the inclusion of sugar alcohols affects the mechanical properties of gelatin gels. This inclusion is mainly known to increase the gelling/melting temperature, and gel strength of gelatin gels (Gekko and Koga, 1983; Gekko et al., 1992; Kasapis et al., 2003; Oakenfull and Scott, 1986; Wang and Hartel, 2022b). Furthermore, it has been reported that the inclusion of polyols improve the long-term storage stability of bovine gelatin gels (Baydin et al., 2022). The amount of polyols possible to include in a product may be limited by the solubility of these compounds. The solubility of sugars and sugar alcohols in water increases with increasing temperatures, and precipitation/crystallization may take place at low temperatures (Marie and Piggott, 2013). The solubility of sorbitol, sucrose, and xylitol in water at 25 °C is 235 g, 210 g, and 200 g per 100 ml water, respectively (Bubnik and Kadlec, 1995; Marie and Piggott, 2013). In gelatin-based confectionery and nutraceutical products, combining multiple sugars or sugar alcohols is common practice (Rapaille et al., 2003). Including less amount of each sugar alcohol, reduces the risk of exceeding the limit of solubility of individual compounds in food systems (Hartel and Shastry, 1991). For confectionery and gummies with high co-solute (sugar alcohols or sugars) content, the processability of gelatin gels is dependent on the viscosity of the gel or gel solution during production (Schrieber and Gareis, 2007).

Naturally occurring organic acids, e.g., citric acid, malic acid, acetic acid, and their salts are commonly used in non-chocolate confectionery to provide sourness and adjust the pH of the product (Burey et al., 2009; Da Conceicao Neta, Johanningsmeier and McFeeters, 2007). Gelatin-based confectionery and gummies generally have a pH value of 3–5 since a pH value below 4.6 is considered by the U.S. Food and Drug Administration as an acid food that prevents the growth of certain bacteria (Burey et al., 2009; FDA, 2018). On the other hand, lower pH values are undesirable in gelatin-based food products because of the acid hydrolysis of peptide bonds in the gelatin backbone, further impacting textural stability (Koli et al., 2013; van den Bosch and Gielens, 2003).

Sugars and sugar alcohols are widely used in the confectionary industry. However, studies comparing their impact on the rheological properties and storage stability of WWFG based gels have not been published, to the authors' knowledge. The scope of this study was to analyze the impact of xylitol, sorbitol, and sucrose on the short-term and long-term stability of WWFG gels. Different co-solutes (sorbitol, xylitol, and sucrose) were included in gelatin gels at a constant concentration (52% (w/w)). In addition, combinations of two different sugar alcohols at two concentrations (52% (w/w) and 66% (w/w)) were studied. The pH value of all gels was kept constant at 4.5 as a compromise by the FDA recommendation and potential extensive acid hydrolysis. The storage stability of the gels was investigated up to 16 weeks. In addition, the processability of the fish gelatin gels with different co-solutes were evaluated regarding their viscosity. The results were discussed from an industrial application point of view.

2. Materials and methods

2.1. Materials

The warm water fish gelatin (200 Bloom) was produced from raw material obtained from iridescent shark (*Pangasianodon hypophthalmus*) and tilapia (*Oreochromis niloticus*). The product was provided by Lapi Gelatine, Italy. Xylitol was provided by Danisco, UK. Sorbitol was purchased from Food Innovation, Norway. Malic acid (MA) and trisodium citrate dihydrate (TCD) were provided by MerckMillipore, USA. Sucrose was purchased from Sigma, USA.

2.2. Composition and preparation of the gels

The concentration of the buffer salts, TCD and MA, was optimized to obtain a stable pH value close to 4.5. The composition of the gels is given in Table 1. The gelatin/water ratio was kept constant at 0.5 among all gels.

The temperature during the preparation of the gels was kept constant

Table 1
The abbreviations and compositions of the fish gelatin gels with different co-solutes. All ingredients are given as (w/w) percent.

Gel name	Fish gelatin (%)	Sorbitol (%)	Xylitol (%)	Sucrose (%)	d-H_2O (%)	TCD (%)	MA (%)
Control (CO)	32.34	–	–	–	64.66	2.00	1.00
Sorbitol (SO)	15.00	52.00	–	–	30.00		
Xylitol (XY)	15.00	–	52.00	–	30.00		
Sucrose (SU)	15.00	–	–	52.00	30.00		
Mix1 (M1)	15.00	26.00	26.00	–	30.00		
Mix2 (M2)	10.33	33.00	33.00	–	20.66		

at 70 °C. Fish gelatin, TCD, and MA were mixed with de-ionized water (18.2 MΩcm Stakpure OmniaPure, Germany) (with 0.02 (w/w)% sodium azide (NaN_3, BDH, UK)) for 30 min with magnetic stirring. 0.05 (w/w)% Witafrol was added to prevent foaming and the mixture was degassed twice with a diaphragm vacuum pump (Vacuubrand, MZ 2C). Any water loss was readded after degassing. The pH of the gel was measured with a pH combination electrode (A 162 2M DI, SI Analytics, Germany) connected to the titrator (7000-M1/20, TitroLine, SI Analytics, Germany) and 5 M HCl was manually titrated until a stable pH of 4.5 was reached. For gels other than the control gel (CO), sorbitol, xylitol, and sucrose were added to the pH optimized gel, and mixed for 20, 15, and 30 min, respectively. For the gels containing both sorbitol and xylitol, sorbitol was added first. After the co-solutes were fully dissolved, the gels were degassed as described above to eliminate air bubbles.

After preparation, a portion of each gelatin solution was kept in the melted state for 20 h in a 55 °C water bath. This protocol is referred to as a short-term stability test in this study. For the long-term stability test, the gels were placed in glass containers immediately after preparation, and the lids were tightly covered with parafilm to prevent evaporation during storage. The containers were immediately placed in ambient temperature, i.e., an average of 22 °C laboratory environment, 30 °C or 40 °C temperature controlled chambers. A setting test was conducted where a gel was split into two glass containers after preparation. The containers were sealed as described above. One container was placed in 30 °C or 40 °C temperature controlled chambers while the gels were still in the sol state after preparation. The other containers were placed at ambient temperature for 24 h and the gels were allowed to set. After 24 h, set gels were placed in 30 °C or 40 °C temperature controlled chambers and were subjected to the long-term stability test similar to the first group. Both groups were analyzed with SAOS measurements as described in Section 2.4.

2.3. a_w measurements

The a_w of the gels was measured with HygroPalm HC2-AW (Rotronic, Switzerland) after the gels were allowed to set for at least 24 h at ambient temperature. The sample was placed into the measurement chamber and the a_w was recorded after 15 min.

2.4. SAOS measurements

Rheological analyses on the gels were performed: i) immediately after gel preparation (t_0), ii) after the short-term stability test (t_{20}), and iii) at selected time points during the storage of the gel (week 4, 8, and 16). The protocol previously reported by Baydin et al. (2022), was followed. SAOS measurements were performed with a rheometer (Malvern Kinexus ultra+, Westborough, USA). The CP4/40 40 mm diameter 4° angle cone and 50 mm diameter sandblasted lower plate with curved edges, supplied by Malvern were used. At selected time points, approximately 2 g of gel sample was placed on the pre-heated lower plate (60 °C) and allowed to melt. The rheometer was operated in 0.1% shear strain-controlled mode, and the frequency was set to 1 Hz. The chosen strain was confirmed to be within the linear viscoelastic region for all samples. To avoid evaporation, the gel sample was covered with silicone oil (10 cS fluid, Dow Corning, UK) prior to and during the measurement. The viscoelastic properties of the sample were obtained using a temperature gradient of 2 °C/min, with a starting and an ending temperature of 60 °C and holding time of 15 min at 20 °C. The highest storage modulus during curing (G'_{max}) and the percent change in G'_{max} ($\Delta G'_{max}$) compared to its t_0 value were calculated using rSpace for Kinexus© software version 1.75 and Microsoft Excel (version 2201). T_t was calculated as the numerical average of T_m and T_g, which were calculated from the temperature where the phase angle corresponded to 45° during heating and cooling, respectively, under the current gelling regime and temperature gradient. ΔT_t is calculated as the change in T_t compared to the T_t value at t_0.

2.5. Viscosity sweep

The viscosity sweep was performed with a Malvern Kinexus ultra + rheometer (Westborough, USA), using a 50 mm diameter 2° cone probe (CP2/50) and a flat bottom plate. Approximately 2 g of pre-melted gel sample was placed on the pre-heated lower plate (50 °C). In order to avoid evaporation, a solvent trap was used during the measurement. The initial test temperature was 40 °C, with following test temperatures of 50 °C, 60 °C, and 70 °C. For each temperature, the sample was first rested for 10 min, i.e., zero shear. Then, a viscosity sweep from 0.01 s^{-1} to 100 s^{-1} was performed, immediately followed by a sweep from 100 s^{-1} to 0.01 s^{-1}. The results were analyzed using rSpace for Kinexus© software version 1.75.

2.6. Texture profile analysis

Texture profile analysis (TPA) test was carried out with TA.XT plusC Texture Analyser (Stable Micro Systems Ltd., UK). The gels were cast using cylindrical metal molds of standard dimensions (20 mm height, 18 mm diameter). The gels were cured at ambient temperature for 48 h prior to analysis. A 30 kg load cell and P/50 50 mm diameter cylinder aluminum probe supplied by Stable Micro Systems Ltd. Were used. Pre-test, test, and post-test speeds were 2 mm/sec. 75% strain was applied. Contact time was 5 s, and trigger force was 5 g. Standard TPA parameters were calculated using Exponent Connect© software (version 7.0.3.0).

2.7. SEC-MALS

The molecular weight distributions of the fish gelatin in the gels were analyzed using size exclusion chromatography coupled with online multiangle laser light scattering (SEC-MALS). Samples were collected from fish gelatin gels at t_0 and after 8 weeks of storage at 30 °C and 40 °C. Sample preparation and SEC-MALS analyses were performed following the protocol reported in Baydin et al. (2022). At week 8, gel samples were frozen in Eppendorf tubes at -30 °C for further analysis. The gel was diluted with elution buffer (0.1 M Na_2SO_4, pH 9 with 0.02 M EDTA), the mixture was heated to 60 °C, and further diluted with elution buffer to obtain a gelatin concentration of 2 mg/ml in each sample. The mixture was filtered with a syringe filter (0.45 μm, Millex-HP, Cork, Ireland) and stored in an HPLC vial (VWR, 1.5 ml, Darmstadt, Germany) at -30 °C prior to analysis. To represent a t_0 sample for the gels, a fresh

gelatin solution was prepared and referred to as undegraded gelatin (UG). UG was treated prior to SEC-MALS analysis as described above for the gelatin gels.

SEC-MALS was performed on an HPLC consisting of a solvent reservoir, online degasser (1260 Infinity degasser, Agilent), Isocratic Pump (1260 Infinity II, Agilent), and automatic sample injector (1260 Infinity II Preparative Autosampler, Agilent). The samples were separated on serially connected OHpak LB-G 6B guard, OHpak LB 803 and OHpak LB 802.5 columns (Showa Denko Europe GmbH, Germany). The sampler and column oven temperatures were 30 °C and 40 °C, respectively. The column outlet was connected to a DAWN HELEOS-II MALLS photometer (Wyatt Technology, USA) ($\lambda_0 = 663.8$ nm) and a Shodex RI-501 refractive index detector. The eluent was 0.1 M Na_2SO_4 with 0.02 M EDTA (pH 9), delivered at a flow rate of 0.5 ml/min. Each sample was analyzed twice with injection volumes of 50 µl and 100 µl. Pullulan (137 kDa) was included as the standard, and dextran (9.89 kDa) was used for normalization, with 100 and 500 µg injected, respectively. Data were collected and processed (with dn/dc = 0.190 ml/g for gelatin) using Astra software (version 7.3.2.21, Wyatt Technology, USA).

2.8. Statistical analysis

Each gel was prepared in two independent replicates, with the exception of CO having three replicates. All statistical analyses were performed using IBM® SPSS® Statistics software version 28.0.1.0 (142). One-way analysis of variance (ANOVA) with post-hoc Tukey's honest significant difference (HSD) test was performed for comparisons of weight average molecular weight ($\overline{M_w}$) and number average molecular weight ($\overline{M_n}$) between groups (gels). Two-way ANOVA with Tukey's HSD with temperature (ambient, 30 °C and 40 °C) as the second factor was conducted for comparisons of ΔT_t and $\Delta G'_{max}$ between groups. Statistically significant differences were reported if $p < 0.05$.

3. Results and discussion

During the production of gelatin-based confectionery, hard shell capsules and nutraceuticals, it is common practice to keep the liquid mass at an elevated temperature for up to 24 h in a holding tank, until it is further processed, gelled and packaged (Burey et al., 2009; Schrieber and Gareis, 2007). In order to simulate a typical production process, the short-term stability of fish gelatin with different co-solutes was tested at 55 °C. CO showed approximately double the percent decrease in G'_{max} compared to gels with co-solutes (SO, XY, SU, M1), whereas minimal differences were observed between the $\Delta G'_{max}$ of different co-solute gels (Supplementary Fig. 1). At t_0, CO had the lowest T_t, whereas co-solute gels had similar T_t values with SU having a slightly lower T_t than the sugar alcohol gels (Supplementary Table 1). All gels exhibited a similar decrease at t_{20}. The short-term stability tests suggested increased stability of fish gelatin gels with the inclusion of co-solutes, without obvious differences between different sugar alcohols or sucrose.

The a_w of the fish gelatin gels with different co-solutes is shown in Table 2. As expected, CO (64.44 (w/w)% water, no co-solute) had the highest and M2 (20.66 (w/w)% water, 66 (w/w)% co-solute) had the lowest a_w, caused by the differences in solvent to co-solute ratio between these gels. SO, XY, SU, and M1 gels contain the same weight amount of water (and co-solutes). The a_w of SU was higher than those of the gels with sugar alcohols. This can be attributed to the higher molecular weight of sucrose (M_w: 342.3 g/mol) compared to sorbitol (M_w: 182.2 g/mol) and xylitol (M_w: 152.2 g/mol) (Favetto and Chirife, 1985; Rahman, 2009). This gives lower molality of sucrose in SU than sorbitol (in SO) or xylitol (in XY) since the gels contain the same concentration (w/w) of co-solutes. The impact of molar concentration on a_w can also be observed for SO and XY with sorbitol having 16.5% higher M_w than xylitol.

Table 2
a_w of fish gelatin gels with different co-solutes. The abbreviations and compositions of the gels are given in Table 1.

	CO	SO	XY	SU	M1	M2
a_w	0.95	0.79	0.76	0.84	0.78	0.63

3.1. Long-term stability: Effect of different co-solutes

The long-term storage stability of fish gelatin gels containing a single co-solute (52 (w/w)%), i.e., either one of two different sugar alcohols (SO, sorbitol and XY, xylitol) or a non-reducing sugar (SU, sucrose), was compared at ambient temperature, 30 °C, and 40 °C. The stability of these gels was compared to the control gel (CO) without any co-solutes. As expected, gels containing co-solutes had higher T_t values compared to CO at t_0 (Choi and Regenstein, 2000; Eysturskard et al., 2010; Gekko and Koga, 1983; Joly-Duhamel et al., 2002; Kuan et al., 2016; Miyawaki et al., 2015; Oakenfull and Scott, 1986). SU had approximately 1.5 °C lower T_t compared to the sugar alcohol gels. None of the gels exhibited a significant decrease in T_t at ambient temperature after 16 weeks. However, as expected, the decrease became significant at elevated storage temperatures (Table 3). The T_t of M1 and M2 will be discussed in Section 3.2.

The ΔT_t of the gels (CO, SO, XY and SU) at all three storage temperatures and time points (4, 8 and 16 weeks) were compared. 40 °C week 16 data point was excluded from the analysis for all gels since no transition temperature could be obtained for CO (Table 3). The results indicated statistically significant differences only between the ΔT_t of CO and SO ($p = 0.026$). Slower gelling and melting kinetics have been reported for mammalian gelatin in the presence of co-solutes (Baydin et al., 2022; Wang and Hartel, 2022b). This was also observed in this study with fish gelatin gels (Supplementary Fig. 2). Although co-solutes increased the initial T_t of the system, they did not contribute to its conservation over time.

At t_0, the average G'_{max} of gels with constant concentration of co-solutes was in the following order: SO > XY > SU (data not shown). The $\Delta G'_{max}$ of the gels after 16 weeks of storage at ambient temperature, 30 °C and 40 °C are shown in Fig. 1A, B and 1C, respectively. At all three storage temperatures, CO showed a larger relative decrease in G'_{max} compared to the gels with co-solutes. Two-way ANOVA was conducted on the $\Delta G'_{max}$ (%) values of the gels with temperature as the second factor. The results indicated statistically significant differences in the means of the gels ($p < 0.01$). Although SU showed slightly lower $\Delta G'_{max}$ compared to SO and XY, Tukey's post-hoc test indicated that only CO presented statistically significant differences compared to the co-solute gels.

SU had a slightly lower T_t and higher a_w compared to the sugar alcohols gels (SO and XY). In a previous study, the positive impact of sorbitol on the structural stability of fish gelatin was found to be superior compared to sucrose. Sugar alcohols may impact the water structure and push gelatin into a more ordered confirmation than sucrose. This could be attributed to greater conformational flexibility and solubility of sorbitol in water, resulting in more cross-links in gel structure (Kamer et al., 2019).

In order to investigate the impact of co-solute type with regards to changes in the molecular weight during storage, SEC-MALS analyses were performed on gelatin from CO, SO, XY, and SU at t_0 and after 8 weeks. Percent change of $\overline{M_w}$ ($\Delta\overline{M_w}$) and $\overline{M_n}$ ($\Delta\overline{M_n}$) at 30 °C and 40 °C are shown in Fig. 2 and Supplementary Fig. 4, respectively. The absolute values of $\overline{M_w}$ and $\overline{M_n}$, measured after 8 weeks storage at 30 °C and 40 °C are shown in Supplementary Figs. 3A and 3B, respectively. As expected, the decrease in $\overline{M_w}$ and $\overline{M_n}$ was larger at 40 °C compared to 30 °C (Baydin et al., 2022). At both temperatures, there was a larger decrease in the average molecular weights for the CO gel compared to the gels with co-solutes. However, a clear difference between the co-solute gels

Table 3
T_t (°C) of fish gelatin gels stored at ambient temperature (A), 30 °C or 40 °C. The abbreviations and compositions of the gels are given in Table 1. The values are the average of two independent replicates. N/A (not available) indicates that no transition temperature could be determined as the solution did not solidify under the current gelling regime and temperature gradient.

Weeks	CO			SO			XY			SU		
	A	30 °C	40 °C	A	30 °C	40 °C	A	30 °C	40 °C	A	30 °C	40 °C
0 (t_0)	25.9	25.9	25.9	34.0	34.0	34.0	33.9	33.9	33.9	32.5	32.5	32.5
4	26.0	25.1	23.3	34.6	34.3	31.9	34.5	33.8	31.3	32.8	32.5	29.9
8	26.0	24.4	22.0	34.6	33.7	29.7	34.0	33.2	29.3	32.6	32.0	27.8
16	25.7	23.3	N/A	34.0	32.8	27.0	33.7	32.5	26.5	32.4	31.4	25.2
Weeks	M1			M2								
	A	30 °C	40 °C	30 °C	40 °C							
0 (t_0)	33.9	33.9	33.9	35.8	35.9							
4	34.5	34.1	31.7	35.8	34.3							
8	34.1	33.4	29.5	35.5	32.5							
16	34.0	32.7	26.8	34.5	29.8							

was not observed. In order to investigate if such a difference statistically exists, one-way ANOVA analyses were conducted on $\Delta\overline{M_w}$ and $\Delta\overline{M_n}$ of CO and the co-solute gels after 8 weeks of storage at 30 °C and 40 °C. At 30 °C, the $\Delta\overline{M_w}$ of CO was significantly different from the co-solute gels, in addition to differences between SO and XY. However, SU was not significantly different from SO and XY (Fig. 2).

After 8 weeks of storage at 40 °C, $\Delta\overline{M_w}$ showed significant differences between gels (CO, SO, XY, and SU) ($p < 0.001$), with CO having a larger decrease than the co-solute gels (Fig. 2). At both 30 °C ($p = 0.332$) and 40 °C ($p = 0.051$), $\Delta\overline{M_n}$ did not show significant differences (Supplementary Fig. 4). The changes in the average molecular weight of M1 and M2 will be discussed in Section 3.2. Although co-solutes contributed to prevent degradation of gelatin and improved the long-term storage stability of the fish gelatin gels, the results point out to comparable effects by the addition of sorbitol, xylitol, or sucrose. It was suggested that co-solutes containing a large number of OH groups may have stronger stabilizing effects on the structure of water surrounding the sugar molecules due to their higher solubility in water (Uedaira et al., 1989). Although sucrose has more OH groups on a single molecule level compared to the sugar alcohols, the sugar alcohol formulations contain a larger amount of total OH groups, considering the molar concentration of the co-solutes.

The gels were visually inspected for any discoloration throughout their long-term storage. A slight color change was observed for CO and SU, starting from week 4. No browning was observed for the polyol gels, due to the absence of reducing groups. However, browning was observed for SU, although only at 40 °C, starting from week 8 (Fig. 3). Sucrose is a non-reducing sugar; therefore, significant browning was not expected for SU. This may suggest that inversion of sucrose takes place during storage at 40 °C and pH 4.5, in the presence of organic acids (Burey et al., 2009). Both elevated temperatures and low pH promote the sucrose inversion reaction (Wang and Hartel, 2022a). For example, at 40 °C and pH 2, 99.9% of sucrose is inverted to glucose and fructose in 209 h (Hartel et al., 2018). Therefore, SU may contain free glucose and fructose which may be subjected to the Maillard reaction, resulting in browning. Alternatively, the sucrose used may contain trace amounts of reducing sugars from the production process. In addition to changes in color, browning may also impact the flavor profile of the gel (Hartel et al., 2018; Lund and Ray, 2017).

If setting of the gelatin gel before climate chamber incubation could increase the stability compared to a direct transfer of the liquid gelatin was investigated. This was tested by performing a setting test (Section 2.2). Paired samples *t*-test was performed on the G'_{max} of the gels from all storage temperatures and time points, comparing the means of gels which were allowed to gel before they were stored at 30 °C or 40 °C, and gelatin solutions which were placed in the temperature chambers immediately after preparation while still liquid. The results indicated no statistically significant difference between the two groups ($p = 0.208$). Therefore, the initial formation of ordered triple helix structures does not seem to be crucial for maintaining the elastic properties of the gel.

3.2. Effect of sugar alcohol concentration

As highlighted in the previous section, an improved storage stability of fish gelatin gels was obtained with the inclusion of co-solutes. To investigate the effect of total sugar alcohol (sorbitol and xylitol) concentration on the long-term storage stability of fish gelatin gels, the T_t and $\Delta G'_{max}$ of CO (no sugar alcohols), M1 (52 (w/w)% sugar alcohols), and M2 (66 (w/w)% sugar alcohols) were compared after storage at 30 °C and 40 °C for 16 weeks. The T_t of the gels were in the following order: M2 > M1 > CO at t_0 and after 16 weeks of storage at all three storage temperatures (Table 3). Statistical tests indicated significant differences in the mean ΔT_t of the groups at two different temperatures (30 °C and 40 °C), excluding 40 °C week 16 measurement for all groups since no transition temperature could be obtained for CO (Table 3). The post-hoc test showed significant differences between CO and M2 ($p = 0.018$), whereas M1 was not significantly different from the other two groups. It has been shown that increasing polyol concentration increases the T_t of gelatin gels (Wang and Hartel, 2022b). The gelling and melting kinetics of the gels became slower as the sugar alcohol concentration increased, indicating that polyols may impede the rearrangement of gelatin molecules (Supplementary Fig. 2).

In accordance with literature, the initial G'_{max} of fish gelatin gels decreased with increasing polyol concentrations (data not shown) (Cai et al., 2017; Kamer et al., 2019; Kuan et al., 2016). Although gelatin/-water ratio was kept constant among gels, gelatin/water phase ratio decreases with increasing sugar alcohol concentrations since polyols dissolve in water, increasing the total volume of the water phase. Moreover, the current temperature gradient may be too rapid for the gelatin molecules to sufficiently realign in the presence of sugar alcohols. The slope of G' during curing decreased with increasing polyol concentrations, suggesting that sugar alcohols may hinder the structural rearrangement of the gelatin network (Supplementary Fig. 2). The decrease in gel strength in the presence of polyols was suggested to be due to high solution viscosity, hindering the motion of gelatin chains (Kamer et al., 2019). At high concentrations, sugar alcohols have been reported to inhibit gelling of gelatin solutions, possibly preventing the formation of stable cross-links (Kamer et al., 2019; Wang and Hartel, 2022b).

CO showed the largest percent decrease in G'_{max}, whereas M2 had the smallest decrease after storage at 30 and 40 °C (Fig. 4). Statistical tests were performed on the $\Delta G'_{max}$ (%) of the gels at two storage temperatures. Significant differences were found between CO and the sugar alcohol gels ($p < 0.001$). However, there was no significant difference

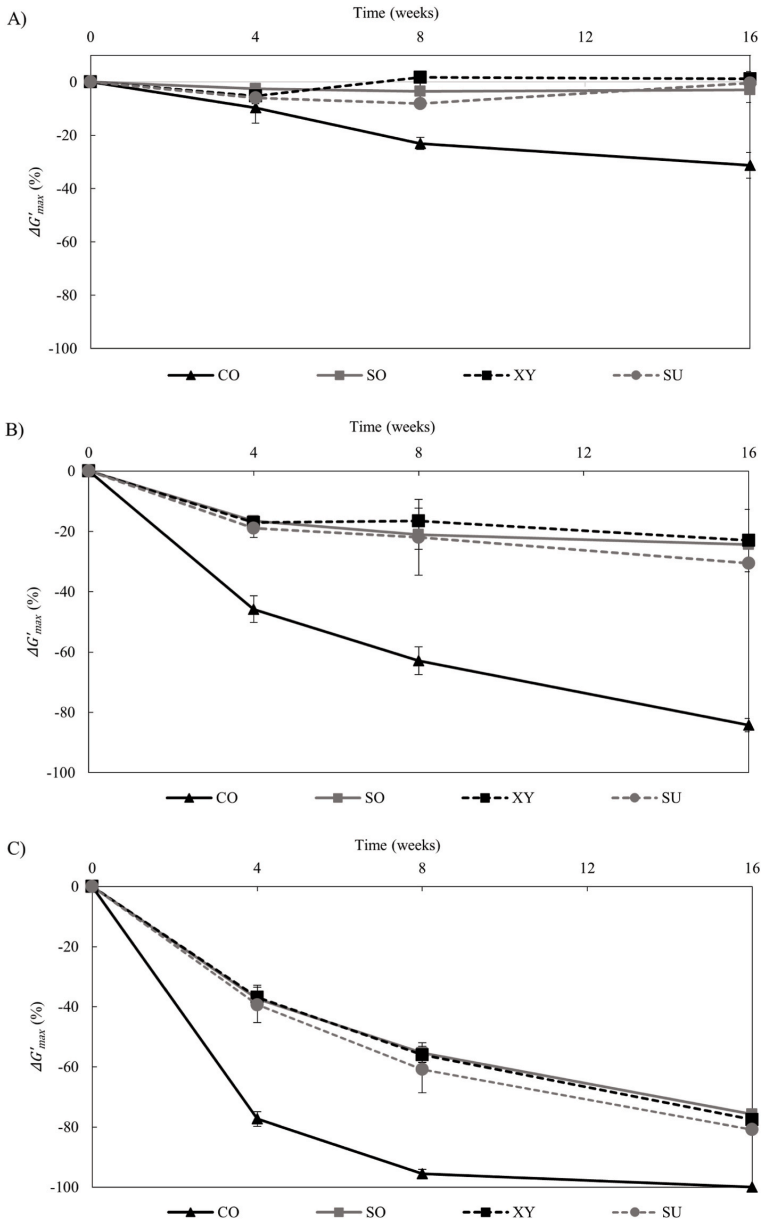

Fig. 1. $\Delta G'_{max}$ of fish gelatin gels with different non-reducing sugars (52 (w/w)%) during storage at A) ambient temperature, B) 30 °C, and C) 40 °C up to 16 weeks. The G'_{max} values are the average of independent replicates (three for CO, and two for SO, XY, and SU), and the error bars indicate standard deviation. The data points are connected to guide the eye. The abbreviations and compositions of the gels are given in Table 1.

between M1 and M2. It should be noted that over time M1 showed a similar decrease in T_t and G'_{max} as SO, XY, and SU (Table 3, Fig. 1), attributed to a similar total co-solute concentration.

The ability of the fish gelatin gel to maintain its elastic properties over time increased with increasing concentrations of sugar alcohols in the formulation, possibly due to lower a_w and higher degree of ordering at elevated temperatures. There are varying theories on the molecular and thermodynamic mechanisms of gel enhancement by sugars and polyols. Naftalin and Symons (1974) suggest that these co-solutes participate in hydrogen bonding between the gelatin chains, promoting and stabilizing the association of gelatin molecules (Eysturskard et al., 2010; Miyawaki et al., 2015). Shimizu and Matubayasi (2014) argue that hydrated co-solutes exclude water from the surface of gelatin molecules leading to increased structural compactness and enhanced aggregation by driving protein-protein interaction. Oakenfull and Scott (1986) suggest that protein stabilization was suggested to be due to

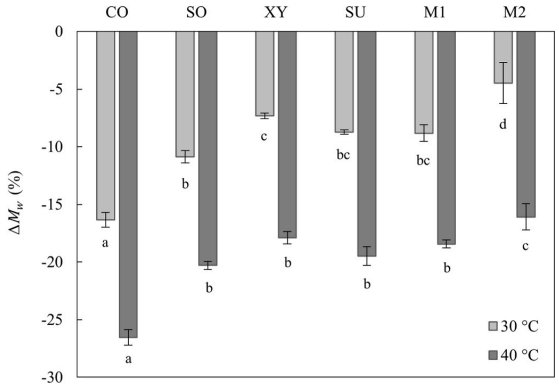

Fig. 2. $\Delta\overline{M_w}$ of fish gelatin gels with different co-solutes after storage at 30 °C and 40 °C for 8 weeks. The results are given as percent change compared to an undegraded gelatin sample. The data points are the average of 2 injections for each sample. Different letters below the light and dark gray columns indicate statistically significant differences ($p < 0.05$) for storage at 30 °C and 40 °C, respectively. The abbreviations and compositions of the gels are given in Table 1.

water-structure-making properties of sugars and sugar alcohols resulting in a preferential hydration of protein, increasing the number of collagen triple helix junction zones (Gekko and Koga, 1983; Gekko et al., 1992). In a recent study, Wang et al. concluded that up to approximately 70% sugars enhance the number of junction zones, whereas higher concentrations suppress it (Kasapis et al., 2003; Wang and Hartel, 2022b). Cai et al. (2017) reported similar results for xylitol preventing gel network formation at increasing concentrations.

SEC-MALS analyses performed on CO, M1 and M2 after 8 weeks of storage at 30 °C did not indicate statistically significant differences for the $\Delta\overline{M_n}$ of gelatins in the systems (Supplementary Fig. 4). However, $\Delta\overline{M_w}$ of the gels were significantly different at this temperature (Fig. 2). The same trend can be observed for the absolute values of $\overline{M_n}$ and $\overline{M_w}$ (Supplementary Fig. 3A). At 40 °C, only M2 showed significantly different $\Delta\overline{M_n}$ compared to CO and M1 (Supplementary Fig. 4). However, all three gels had significantly different $\Delta\overline{M_w}$ at this temperature (Fig. 2 and Supplementary Fig. 3B). The results suggest that increasing sugar alcohol concentrations decreased the degradation of gelatin in the systems both below and above the T_t of sugar alcohol gels.

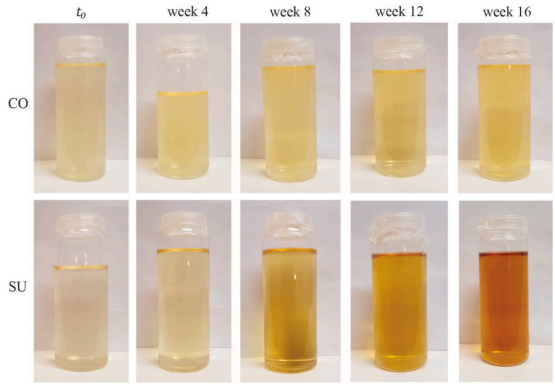

Fig. 3. CO and SU throughout long-term storage at 40 °C. The abbreviations and compositions of the gels are given in Table 1.

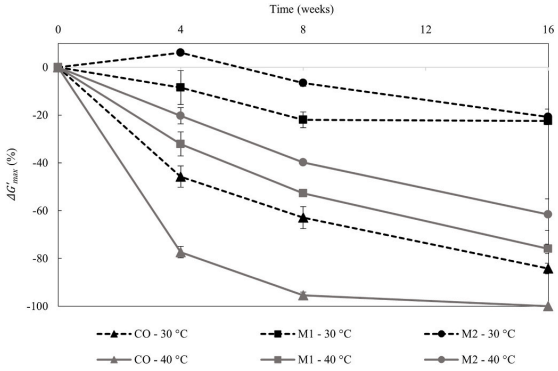

Fig. 4. $\Delta G'_{max}$ of CO (▲), M1 (■) (52% (w/w) polyols), and M2 (●) (66% (w/w) polyols) after 16 weeks of storage at 30 °C (black shapes) and 40 °C (gray shapes). Each data point represents the average independent replicates (three for CO, and two for M1 and M2), and the error bars show standard deviation. The data points are connected to guide the eye.

After preparation, turbidity was observed for M2 after it had set at ambient temperature. However, this was not observed for CO or M1 (Fig. 5). In addition, 24 h after preparation, small water droplets were observed on the surface of M2, indicating syneresis. Since no large-scale crystallization growth was observed, the turbidity did not seem to be caused by the precipitation of sugar alcohols (Hartel and Shastry, 1991). Moreover, photomicrographs indicated differences in the network structure between M1 and M2 (data not shown).

A standard TPA test was performed on M1 and M2. The results indicated similar cohesion and springiness values for both gels (Table 4, Supplementary Fig. 5), suggesting that the turbidity observed for M2 was not due to polyol crystallization. Combined with the syneresis observed for M2, a possible explanation may be increased protein-protein interactions such as further lateral association of triple helices to reach the size of light scattering domains (Shimizu and Matubayasi, 2014). A similar result has been reported for starch solutions in the presence of sugars, where aggregation was suggested to be due to a lower amount of free water available to dissolve the biopolymer, resulting in increased starch-starch interactions resulting from a reduced solvent quality (Heydari et al., 2018). Further testing is required to obtain the full molecular mechanism behind this result. X-ray scattering and circular dichroism may be used to observe the structural differences and to determine at which temperature turbidity starts occurring in the high sugar alcohol system.

3.3. Viscosity

Viscosity is an important property for the commercial processability

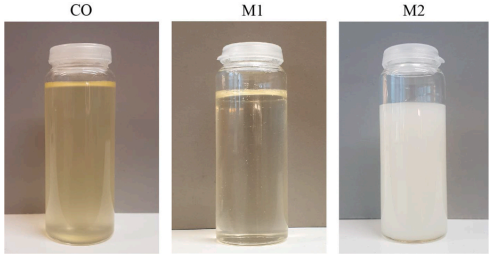

Fig. 5. Photographs of CO, M1, and M2 gelled at ambient temperature. The abbreviations and compositions of the gels are given in Table 1.

Table 4
Texture characteristics of M1 and M2 obtained with standard TPA test. Double compression was carried out with 75% strain. The results are given as the average of 3 replicates ± standard deviation. The abbreviations and compositions of the gels are given in Table 1.

	Hardness (g)	Resilience (%)	Cohesion	Springiness (%)	Gumminess
M1	15,568 ± 518	63.4 ± 0.3	0.86 ± 0.01	96.40 ± 0.33	13,388 ± 240
M2	9739 ± 441	55.2 ± 0.3	0.85 ± 0.00	96.58 ± 0.92	8276 ± 367

of gelatin-based products (Ward and Courts, 1977). For molded products such as confectionery, high viscosity of gelatin solutions will represent challenges in the production process since flowability is crucial (Schrieber and Gareis, 2007). The complex viscosity (η^*) of fish gelatin gels with different co-solutes during the temperature range of SAOS measurements was analyzed. The η^* of the gels at 60 °C was in the following order: SU > M2 > SO > M1 = XY ≫ CO (Supplementary Fig. 6; Supplementary Table 2). This initial analysis showed the impact of the type and concentration of co-solute on the viscosity of the fish gelatin solutions. In addition, the activation energy of fish gelatin solutions before gelling and after re-melting are shown in Supplementary Table 3. In order to further analyze the viscoelastic properties of the solutions, viscosity sweeps were performed at a temperature range from 50 °C to 70 °C. Significant thixotropy was not observed between the first sweep from 0.01 s^{-1} to 100 s^{-1} and from 100 s^{-1} to 0.01 s^{-1}, with all solutions showing Newtonian behavior in the shear rate range tested. Viscosity curves of co-solute gels from 100 s^{-1} to 0.01 s^{-1} are given in Supplementary Fig. 7.

Viscosity curves indicated higher shear viscosity (η) of the gels at lower temperatures, as expected (Poppe, 1992). At 50 °C, the shear viscosity of the gels at 1 s^{-1} shear rate followed the order: SU > M2 > SO > M1 > XY (Fig. 6). Although SU (52 (w/w)%) has a lower concentration of co-solutes than M2 (66 (w/w)%), it had a significantly higher viscosity. SU also had a higher viscosity than the sugar alcohol gels (SO, XY and M1) at all analysis temperatures. Higher viscosity of the solution may require a higher processing temperature to maintain processability during mixing and molding of gelatin-based products with sucrose. Elevated temperatures during processing may also have further consequences, such as increased hydrolysis of the gelatin, or degradation of temperature sensitive ingredients embedded in the product, e.g., vitamins in the case of vitamin gummies. In order to obtain a similar a_w to sugar alcohol gels, the sucrose concentration would need to be increased, causing even higher viscosity than the current SU system. It should be noted that at 40 °C, M2 had even higher viscosity than SU (Supplementary Fig. 7E). Increasing the co-solute concentration in gelatin gels may result in processing challenges during the production, and replacing at least some of the sucrose with low molecular weight sugars or polyols may be beneficial (Schrieber and Gareis, 2007).

Previous research agrees that the addition of sugars or polyols increase the viscosity of gelatin solutions (Cai et al., 2017; Kuan et al., 2016; Miyawaki et al., 2003; Wang and Hartel, 2022b). Wang and Hartel reported that for low moisture gelatin gels with high concentrations of co-solutes, sol-gel transition was hindered due to very high viscosity of the systems (Wang and Hartel, 2022a, 2022b). As previously discussed in Section 3.2, the kinetics of gelation have been suggested to be inhibited by the rearrangement of gelatin chains in the presence of co-solutes due to increased viscosity of bulk water, resulting in suppressed motion of gelatin chains (Bryant and McClements, 2000; Kasapis et al., 1999; Kuan et al., 2016). This is in accordance with slower gelation kinetics observed with increasing concentration of sugar alcohols in this study (Supplementary Fig. 2).

Wang and Hartel (2022b) reported a higher T_t and increased viscosity for gelatin gels with increased sugar content. In our study both the sucrose and sugar alcohol gels exhibited higher T_t and viscosity compared to the control gel without co-solutes. However, with sucrose as compared to sugar alcohol formulations, the viscosity of the system was higher although T_t was lower. This may be attributed to the higher molecular weight, and therefore, the lower molarity of sucrose in the formulation. A smaller number of sucrose molecules bind to water, resulting in an increase in the amount of free water in the system, i.e., increased solvent quality. On the other hand, at the same concentration, a larger number of sugar alcohol molecules will bind to water, decreasing the amount of free water and consequently driving protein-protein interactions in the system (Antoniou et al., 2010; Heydari et al., 2018; Ma and Pawlik, 2007; Sakai, 1968). As discussed in Section 3, the molarity of the co-solute in the solution affects a_w. Miyawaki et al. (2003) reported that a small change in a_w significantly affected the protein-protein interactions through the change in hydration state of gelatin molecules, especially near the T_t. As the a_w of fish gelatin gel decreased with the inclusion of co-solutes, physical properties that are more similar to mammalian gelatin were observed, including improved storage stability (Baydin et al., 2022).

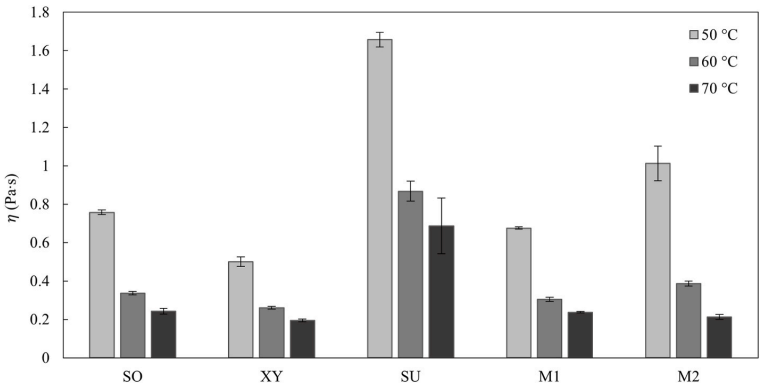

Fig. 6. Shear viscosity (η) of fish gelatin gels with different co-solutes at 1 s^{-1} shear rate. The data is given as the average of two replicate gels and the error bars show the standard deviation. The abbreviations and compositions of the gels are given in Table 1.

4. Conclusions

Long-term stability of fish gelatin gels with different co-solutes could be predicted by the short-term stability tests; the inclusion of sugar alcohols or sucrose contributed positively to the stability of fish gelatin in both gels and solutions. However, the type of co-solute did not have a large impact on stability at constant concentration. The addition of sucrose resulted in high viscosity solutions, potentially requiring high processing temperatures which may result in a larger degree of hydrolysis of the gelatin and degradation of temperature sensitive active ingredients. In addition, the browning observed for the sucrose gel may pose disadvantages for the visual characteristics and flavor profile of the final product. Although storage stability increased with increasing concentrations of sugar alcohols, turbidity was observed with the 66 (w/w)% sugar alcohol gel, suggesting a higher degree of gelatin aggregation at this concentration. Future work is required using methods such as X-ray scattering or circular dichroism in order to clarify the structural changes occurring in the high sugar alcohol system. Lowering the water activity of fish gelatin gels with sugars and sugar alcohols resulted in properties much more similar to mammalian gelatin gels. This can be utilized to replace mammalian gelatin in food applications. Although lowering the water activity provided higher stability, it resulted in higher viscosity gelatin solutions and potential processing challenges with gelatin-based confectionery, nutraceutical, and pharmaceuticals.

Credit author statement

Tuna Baydin: Conceptualization, Methodology, Data curation, Visualization, Writing – original draft, Formal analysis, Validation. **Morten J. Dille**: Validation, Methodology, Data curation, Formal analysis, Writing – review & editing. **Olav A. Aarstad**: Investigation, Methodology, Data curation, Software. **Magnus N. Hattrem**: Project administration, Conceptualization, Supervision, Writing – review & editing. **Kurt I. Draget**: Resources, Project administration, Conceptualization, Supervision, Writing – review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfoodeng.2022.111334.

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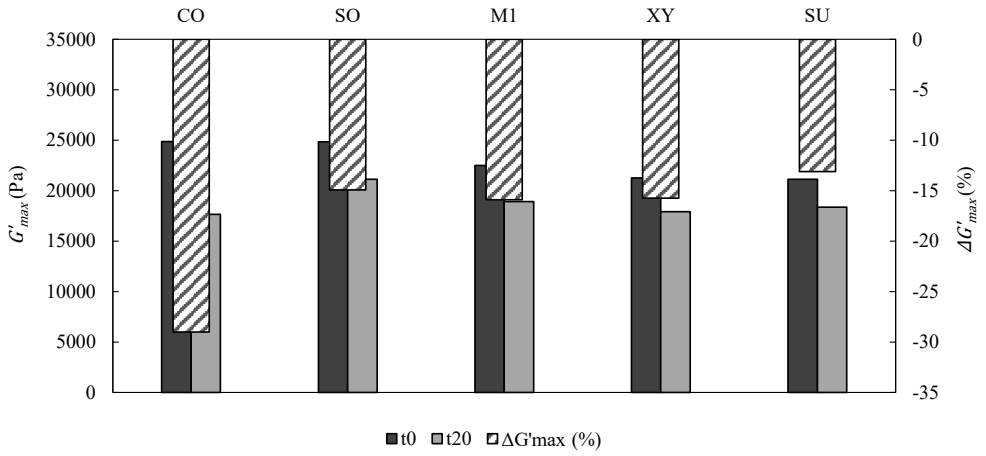
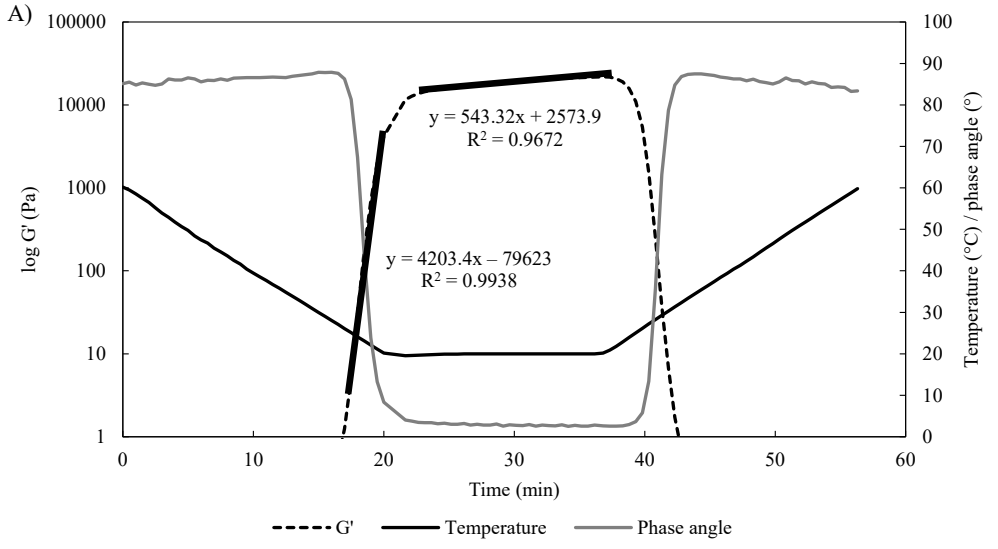
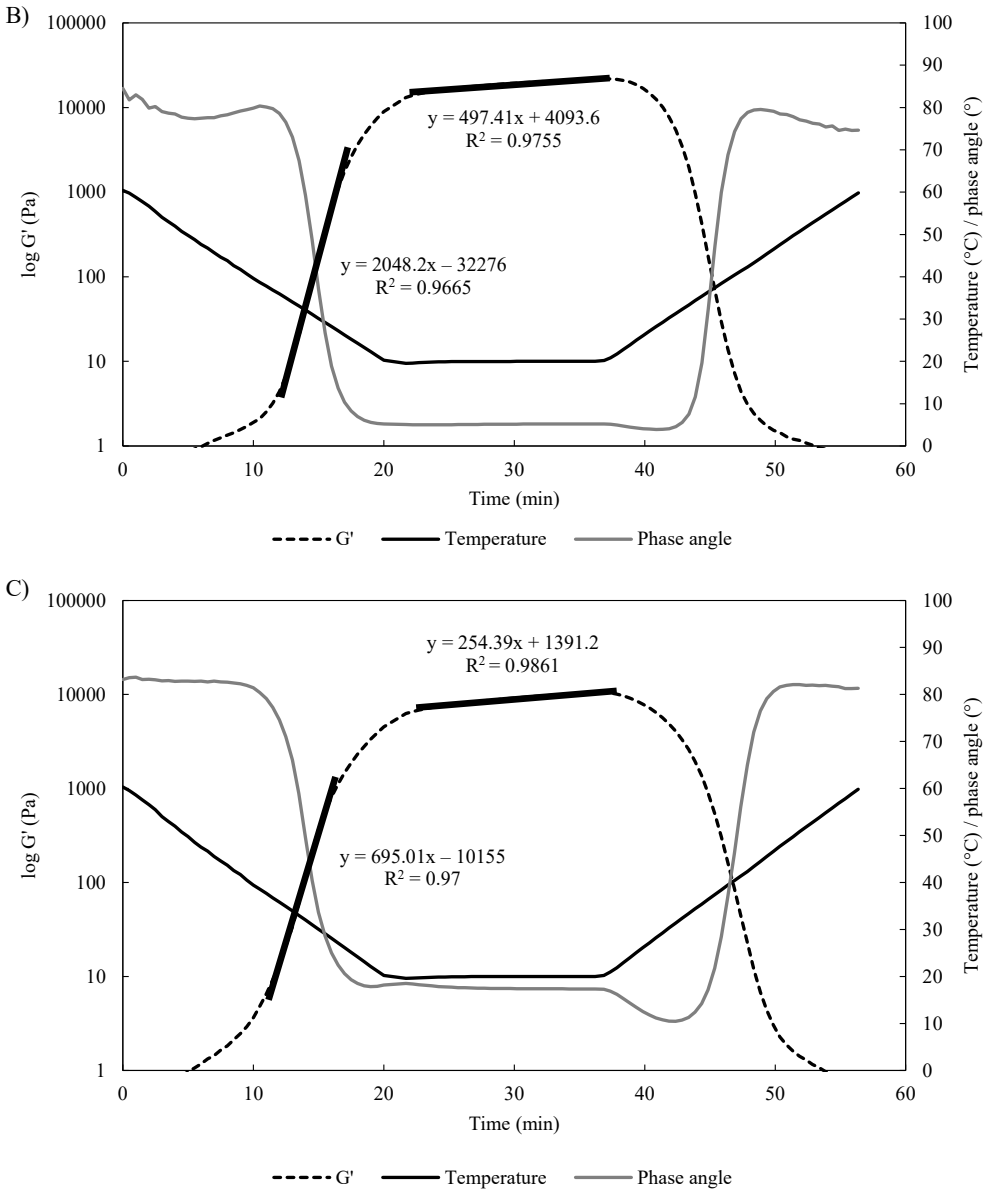
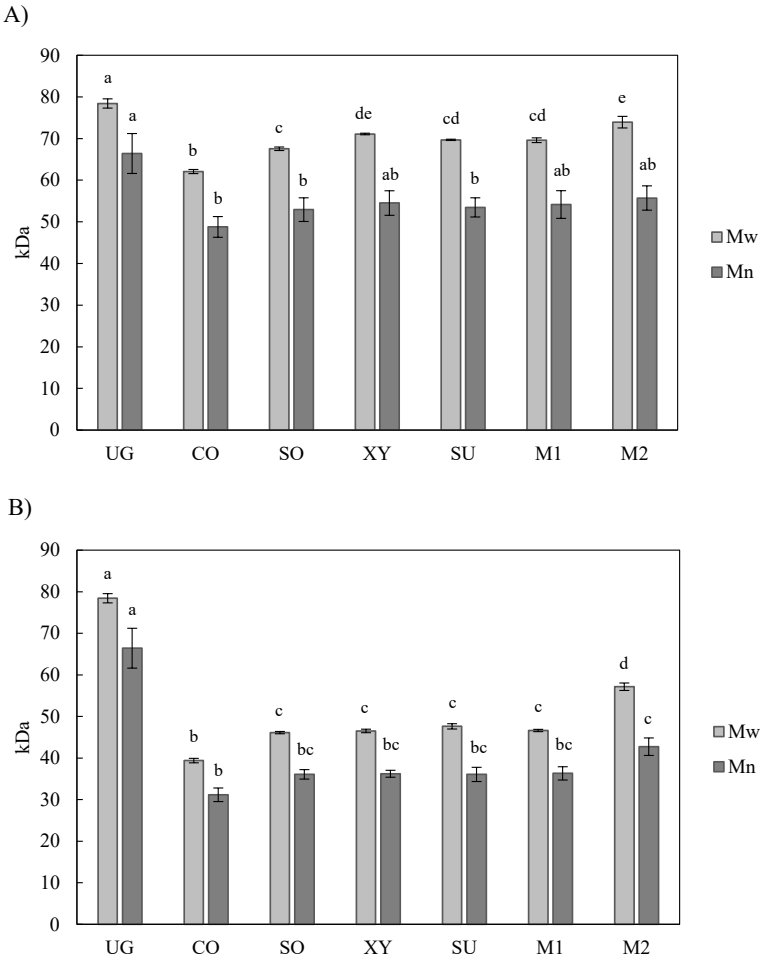


Supplementary Figure 1: Short-term stability test of fish gelatin gels with different co-solutes. The change in G'_{max} after the short-term stability test at 55 °C for 20 hours (t_{20}) are shown. $\Delta G'_{max}$ indicates percent change compared to the gels t_0 value. The abbreviations and compositions of the gels are given in Table 1.

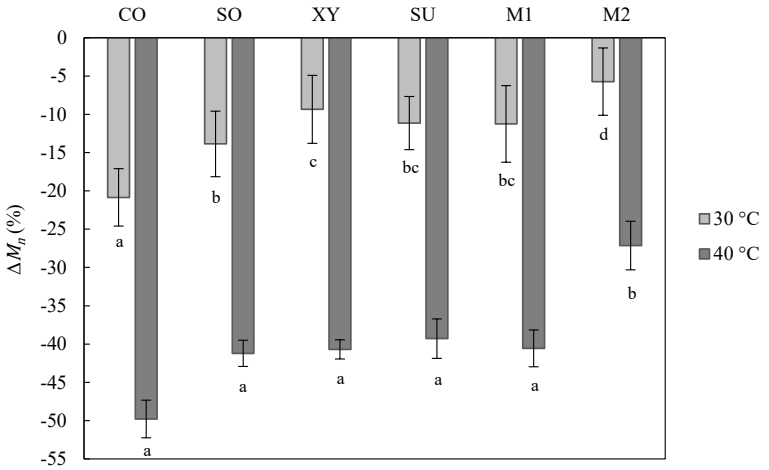




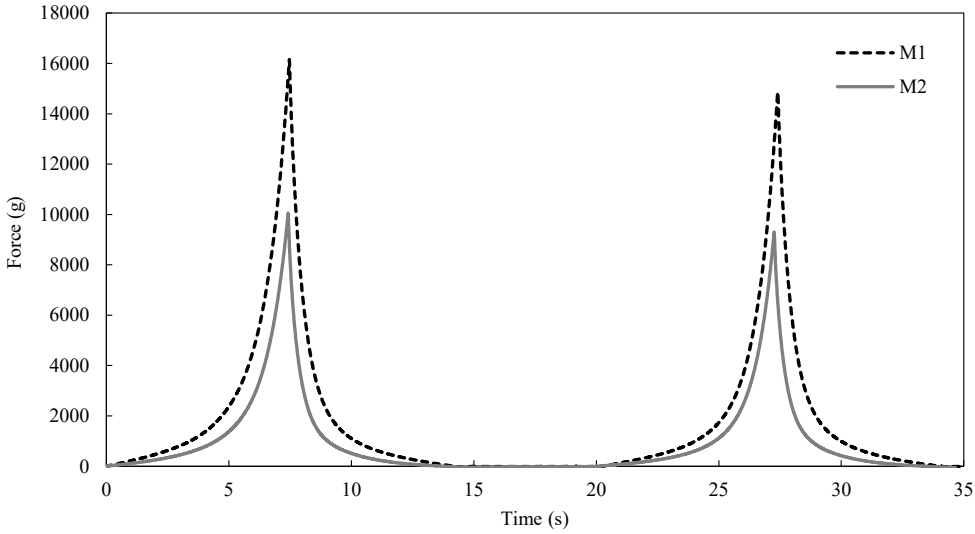
Supplementary Figure 2: Oscillation curves of A) CO, B) M1, and C) M2. The abbreviations and compositions of the gels are given in Table 1.



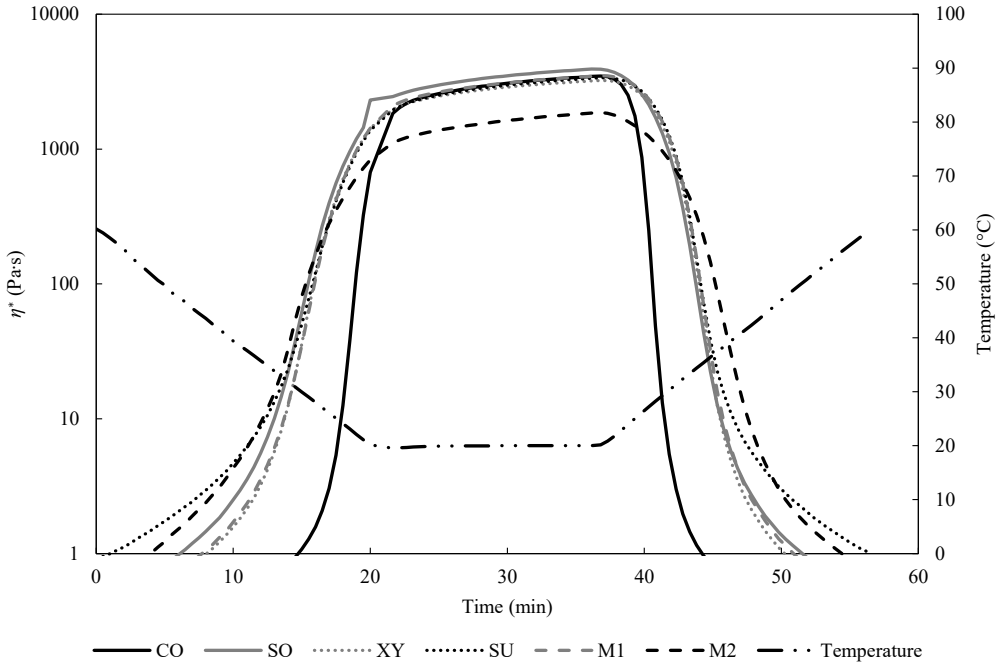
Supplementary Figure 3: $\overline{M_w}$ and $\overline{M_n}$ of undegraded gelatin (UG) and fish gelatin gels with different co-solutes after storage at A) 30 °C and B) 40 °C for 8 weeks. The results are given as $\overline{M_w}$ or $\overline{M_n}$ (kDa) ± standard deviation, n=2 injections were performed for each sample. Different letters above the light and dark gray columns indicate statistically significant differences ($p < 0.05$) for $\overline{M_w}$ and $\overline{M_n}$, respectively. The abbreviations and compositions of the gels are given in Table 1.



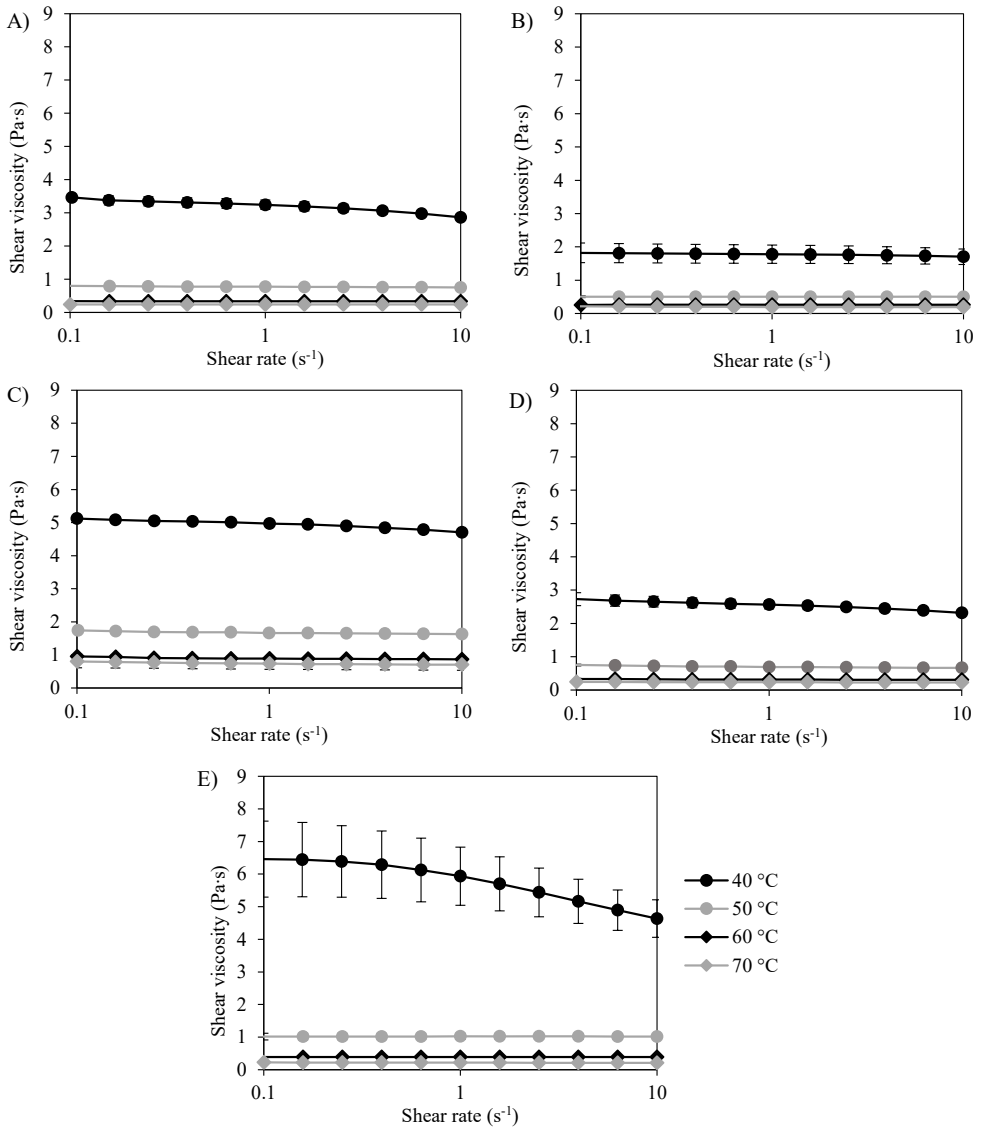
Supplementary Figure 4: $\Delta\overline{M_n}$ of fish gelatin gels with different co-solutes after storage at 30 °C and 40 °C for 8 weeks. The results are given as percent change compared to an undegraded gelatin sample. The data points are the average of 2 injections for each sample, and the error bars show standard deviation. Different letters below the columns indicate statistically significant differences ($p < 0.05$) for storage at 30 °C and 40 °C, respectively. The abbreviations and compositions of the gels are given in Table 1.



Supplementary Figure 5: TPA test on M1 and M2. The abbreviations and compositions of the gels are given in Table 1.



Supplementary Figure 6: Complex viscosity (η^*) of fish gelatin gels with different co-solutes throughout the temperature range measured with small amplitude oscillatory shear measurements. The measurement was performed at 1 Hz (equivalent to a shear rate of 6.28 s^{-1} (Mark, Erman, & Roland, 2013)) and it is assumed that no differences in structuring between the different systems exist (for structures that would be destroyed under continuous shear). The abbreviations and compositions of the gels are given in Table 1.



Supplementary Figure 7: Viscosity sweep of A) SO, B) XY, C) SU, D) M1, and E) M2 at 40, 50, 60, and 70 °C. The abbreviations and compositions of the gels are given in Table 1.

Supplementary Table 1: T_t of fish gelatin gels with different co-solutes at t_0 and after the short-term stability test (t_{20}). The abbreviations and compositions of the gels are given in Table 1.

Gels	T_t	
	t_0	t_{20}
CO	25.9	25.1
SO	34.0	33.0
XY	33.9	32.9
SU	32.5	31.7
M1	33.9	32.9

Supplementary Table 2: Complex viscosity (η^*) of fish gelatin gels with different co-solutes at t_0 and after storage at ambient temperature (A), 30 °C, and 40 °C up to 16 weeks. The values were obtained in the beginning of the temperature range, at 60 °C. The abbreviations and compositions of the gels are given in Table 1.

Weeks	CO			SO			XY			SU		
	A	30 °C	40 °C	A	30 °C	40 °C	A	30 °C	40 °C	A	30 °C	40 °C
0 (t_0)	0.25			0.43			0.33			0.93		
4	0.25	0.19	0.12	0.52	0.57	0.40	0.45	0.36	0.27	1.12	1.16	0.78
8	0.23	0.16	0.08	0.63	0.59	0.23	0.40	0.36	0.17	1.17	1.10	0.47
16	0.22	0.09	0.06	0.54	0.46	0.15	0.41	0.32	0.11	1.23	1.06	0.39

Weeks	M1			M2	
	A	30 °C	40 °C	30 °C	40 °C
0 (t_0)	0.33			0.59	
4	0.47	0.46	0.32	0.77	0.79
8	0.48	0.38	0.21	0.70	0.57
16	0.54	0.37	0.12	0.62	0.35

Paper 3. Plant-based pre-emulsified chewable gels for oral delivery of nutraceuticals

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Physical and functional properties of plant-based pre-emulsified chewable gels for the oral delivery of nutraceuticals

Tuna Baydin [a,b,*], Stine W. Arntsen [a], Magnus N. Hattrem [b], Kurt I. Draget [a,b]

[a] Department of Biotechnology and Food Science, Norwegian University of Science and Technology, Trondheim, Norway
[b] Vitux AS, Oslo, Norway

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ABSTRACT

Gelatin-based pre-emulsified chewable gels have presented advantages over traditional tablets, bulk oils, hard and soft capsules for oral delivery. Ethical, ecological, and religious considerations have increased the demand for plant-based gelling agents which can be formulated into chewable emulsion gels. Plant-based polysaccharide emulsion gels prepared with agar and pectin were compared to gelatin emulsion gels regarding rheological, textural, and functional properties. The agar emulsion gel had higher gelling/melting temperatures (T_g: 40 °C, T_m: 90 °C) than the gelatin emulsion gel (T_g: 37 °C, T_m: 45 °C), whereas pectin emulsion gel had a more similar gelling/melting profile to the gelatin formulation (T_g: 38 °C, T_m: 54 °C). Texture analyses revealed that the agar emulsion gel had a harder and more brittle texture, whereas pectin emulsion gel had a softer texture than the gelatin emulsion gel. Pectin emulsion gels had the largest average droplet size (32 μm), followed by agar (13 μm) and gelatin emulsion gels (1 μm). The *in vitro* lipolysis experiments indicated that the polysaccharide emulsion gels were lipolyzed to a lower extent and had a slower initial lipolysis rate (agar: 2.8 μmol FFA/*sec*, pectin: 4.3 μmol FFA/*sec*), compared to the gelatin emulsion gel (24.9 μmol FFA/*sec*). The industrial potential and challenges of the polysaccharide emulsion gels were evaluated, and the results show that plant based pre-emulsified chewable gels can be manufactured for the oral delivery of nutraceuticals.

PAPER 3

1. Introduction

Food supplements and nutraceuticals are consumed to complement a diet with micronutrients, aiming to enhance health and provide medical benefits (DeFelice, 1995; Santini & Novellino, 2017). Essential fatty acids, vitamins, minerals, and carotenoids are among important nutraceuticals which may be incorporated into an applicable dosage form (Chen, Remondetto & Subirade, 2006; Karuna & Prasad, 2015). Typical oral delivery forms include soft and hard gel capsules, tablets, elixirs, syrups, and chewables (Adepu & Ramakrishna, 2021; Dille, Hattrem & Draget, 2018b). There has been an increasing demand for the development of user-friendly dosage forms, in which a chewable delivery system is considered to be a practical alternative (Dille, Hattrem & Draget, 2018a). The most common chewable delivery forms are similar to gummies/confectioneries in appearance, in which sweeteners, acidulants, aromas and colorants are used to make a well-tasting product. Besides having a good palatability, which is important for compliance, the product also has the uniformity of a tablet, and its chewable texture removes the need for water in the process of ingestion. Although chewable dosage forms offer a user-friendly design, their development and production have been challenging. In the formulation, the water-soluble actives are typically in a solubilized state, while fat-soluble actives need to be mixed throughout the continuous water phase as small oil droplets (Dille et al., 2018a). This may be especially challenging if the payload of the fat soluble active ingredient is high, as mixing of the oil phase requires a more complex production process, while maintaining the stability of the oil phase throughout the manufacturing process. The chemical stability of the embedded ingredient may to a certain extent be ensured by innovative formulation and packaging technology, while to obtain a stable droplet size at elevated temperatures for a longer period of time, the choice of emulsifier, and gelling agent is of high importance.

Emulsion gels *i.e.*, gelled emulsions, are complex soft-solid colloidal materials which consist of an emulsion and a gel (Dickinson, 2012). Emulsion gels can be classified as emulsion droplet-filled gels and emulsion droplet-aggregated gels (Dickinson, 2012; Lin, Kelly & Miao, 2020b). In emulsion droplet-filled gels, a polysaccharide or protein gel constitutes the continuous gel matrix which contains embedded emulsion droplets (Gravelle & Marangoni, 2021). If the emulsion droplets strongly interact with the gel network, the droplets are generally referred to as active fillers. The term inactive filler is used for systems in which the emulsion droplets have little to no interaction with the surrounding gel network (Chen & Dickinson, 1999). In the context of

* Corresponding author.
E-mail address: tuna.baydin@ntnu.no (T. Baydin).

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oral delivery of nutraceuticals, emulsion gels are convenient delivery forms compared to traditional capsules, bulk oils or liquid emulsions which may present compliance issues for pediatric and geriatric populations, as well as patients suffering from dysphagia. In addition, emulsion gels provide physical stability and mechanical properties to emulsions (Lu, Mao, Hou, Miao & Gao, 2019).

Due to their unique texture and emulsifying capability, chewable gelatin-based emulsion gels have been studied, characterized, and patented (Dille et al., 2018a, 2018b; Hattrem, Dille, Seternes, Ege & Draget, 2018, 2015; Haug & Draget, 2007). Gelatin-based emulsion gels typically exhibit an active filler effect, whereas polysaccharide-based emulsion gels made with hydrocolloids with low surface activity typically exhibit an inactive filler effect (Dille et al., 2018a; Koç et al., 2019). One challenge with gelatin-based gels is the low melting temperature of gelatin which may result in the instability of the delivery system in warm climates during transportation or storage of the products (Baydin, Aarstad, Dille, Hattrem & Draget, 2022). Another disadvantage is the animal origin of gelatin since it is obtained from the connective tissues of cattle, pigs, fish or poultry (Schrieber & Gareis, 2007). Ecological, ethical, and health concerns, religious constraints as well as dietary restrictions may endorse vegetarian or vegan diets for individuals (Leitzmann, 2014). In the recent years, there has been an increase in the number of people who follow a plant-based diet (Alcorta, Porta, Tárrega, Alvarez & Vaquero, 2021). This has resulted in an increase in the demand for plant-based food alternatives in the market (Noguerol, Pagán, García-Segovia & Varela, 2021; Stannard, 2018).

Although gelatin is an animal-derived biopolymer, terms such as plant-based or "veggie gelatin" have been used to describe plant hydrocolloids with gelling properties (Alipal et al., 2021; Lestari, Octavianti, Jaswir & Hendri, 2019). Emulsion gels have been prepared with polysaccharides such as agar (Kim, Gohtani & Yamano, 1997, 1996, 1999; Yamano, Kagawa, Kim & Gohtani, 1996), pectin (Lupi et al., 2015), carrageenans (Fontes-Candia, Ström, Lopez-Sanchez, López-Rubio & Martínez-Sanz, 2020; Sala, de Wijk, van de Velde & van Aken, 2008), cellulose (Jiang et al., 2019), and other polysaccharides (Dun et al., 2020; Weiss, Scherze & Muschiolik, 2005; Yang et al., 2019). Although most plant-based hydrocolloids can act as stabilizing agents of emulsions due to structuring, thickening or gelling of the continuous phase, they usually lack the surface active properties to act as emulsifying agents (Dickinson, 2009). Therefore, an emulsifying agent is commonly included in combination with a plant-based gelling agent to provide sufficient emulsifying and stabilizing capacity in such systems (Banerjee & Bhattacharya, 2011; Shao et al., 2020). Another challenge with plant-based emulsion gels is their texture since gelatin has a characteristic elastic, chewy, melt-in-the-mouth texture which is very difficult to mimic with plant-based alternatives (Karim & Bhat, 2008; Schrieber & Gareis, 2007). Other issues with polysaccharide emulsion gels have been reported as stability, syneresis, and the complexity of the systems (Lin, Kelly, Maidannyk & Miao, 2020a; Yue et al., 2022).

In addition to being more practical and user-friendly than traditional oral delivery forms, gelatin-based emulsion gels have been shown to have comparable stability and dissolution kinetics to a standard oral tablet (Dille et al., 2018b). Furthermore, the bioavailability of omega-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) significantly increased when orally delivered in a chewable gelatin-based emulsion gel compared to bulk oil (Haug et al., 2011). Digestibility of food emulsion gels are commonly studied through *in vitro* lipolysis experiments to preliminarily investigate the functionality of formulations. *In vitro* lipolysis studies of gelatin emulsion gels indicated the relation between the gel mesh size and speed of lipid digestion (Sarkar et al., 2015). *In vitro* lipolysis of agar emulsion gels was studied and compared with emulsions without agar present in the formulation, suggesting limitations of digestion due to entrapment of oil droplets in the agar network (Wang, Neves, Kobayashi, Uemura & Nakajima, 2013). Similarly, the lipolysis extent of pectin emulsion gels were lower compared to liquid emulsions (Yang et al., 2022). The methodology and digestive parameters of the *in vitro* lipolysis experiment may have a large impact on the results of the digestion studies (Li, Hu & McClements, 2011; Lin & Wright, 2018; Mella, Quilaqueo, Zúñiga & Troncoso, 2021). Therefore, for comparative analyses, it is of importance to test different formulations in a standardized *in vitro* lipolysis set-up (Li et al., 2011). To the authors' knowledge, a study comparing the extent of *in vitro* lipolysis of emulsion gels prepared with gelatin, agar, and pectin, has not been previously published.

The present study focuses on two polysaccharide-based emulsion gels: agar and pectin. Agars are moderately sulfated galactans, obtained from red seaweed, and are a mixture of agarose and agaropectin (Araki, 1956; Sousa, Rocha & Gonçalves, 2021). Pectins are a group of complex heteropolysaccharides, mainly containing galacturonic acid residues, and are obtained from terrestrial plants (Hua, Wang, Yang, Kang & Zhang, 2015). Commercial pectins may be amidated or methyl-esterified to varying degrees (Da Silva & Rao, 2006; Zeeb, Roth & Endreß, 2021). The scope of this study was to characterize emulsion gels made with polysaccharides, agar, and pectin, and to compare the rheological and textural properties of these gels with a traditional gelatin emulsion gel. In addition, agar and pectin gels that do not contain oil were compared to corresponding emulsion gels to study the impact of oil on the rheology and texture of the gels. Along with the comparison of the physical and textural properties of the gels, their functionalities were studied with gastrointestinal *in vitro* lipolysis experiments. The object of this investigation was to develop plant-based emulsion gels for the oral delivery of nutraceuticals and evaluate their potential as alternatives to gelatin-based emulsion gels.

2. Materials and methods

2.1. Materials

Agar (Gelagar HDR 300) was purchased from B&V, Italy. Bovine gelatin (Type B 160 Bloom, Batch# 643208) was provided by Gelita®, Germany. Xylitol was provided by Danisco, UK. Sorbitol was purchased from Food Innovation, Norway. Malic acid (MA) and trisodium citrate dihydrate (TCD) were provided by MerckMillipore, USA. Corn oil (lot# MKCH1635) and ascorbic acid were purchased from Sigma, USA. Citrem (Grinsted N 12 veg kosher, batch# 4011722438) was purchased from Danisco, Denmark. Hydrolyzed sunflower lecithin (Giralec HE-60) was provided by Lasenor, Spain. GENU® pectin (LM-104 AS-FS) was provided by CP Kelco, Denmark. Calcium citrate tetrahydrate (CCT) was provided by Gadot Biochemical Industries, Israel. Bile extract porcine (lot# SLCC9272) was purchased from Sigma Aldrich, USA. Pancreatin from porcine pancreas (8xUSP, lot# SLBZ5739) and lipase from porcine pancreas (100–500 U/mg, lot# SLBZ7254) were purchased from Sigma Aldrich, USA. Rabbit gastric lipase (RGE15, 15 U/mg lipase and 500 U/mg pepsin, lot# BCBV8659) was purchased from Lipolytech, France.

2.2. Composition and preparation of the gels

Agar gel (AG) was prepared by mixing agar and deionized water (18.2 MΩcm Stakpure OmniaPure, Germany) at 90 °C for 30 min with magnetic stirring. After agar had completely dissolved, 0.06 (w/w)% Witafrol was added, and the solution was degassed using the Diaphragm Vacuum Pump (Vacuubrand, MZ 2C) until no air bubbles were visible. The water loss was compensated after degassing. The solution was allowed to gel at ambient temperature, *i.e.*, an average of 22 °C laboratory environment. Agar emulsion gel without oil (AWO), *i.e.*, agar gel with sugar alcohols and buffer salts, was prepared by first dry mixing agar, sorbitol, and xylitol. Ambient temperature water was added to the powder mixture and the contents were mixed at 90 °C for 30 min with magnetic stirring. The temperature was reduced to 65 °C and MA was added to the mixture and mixed for 10 min. Afterwards, TCD was added to the mixture and mixed for 10 min. Agar emulsion gel (AEG) was prepared in

Table 1
Abbreviations and compositions of the gels. All ingredients are given as weight percentage (w/w).

Ingredient	Function	Agar gel (AG)	Agar emulsion gel without oil (AWO)	Agar emulsion gel (AEG)	Pectin emulsion gel without oil (PWO)	Pectin emulsion gel (PEG)	Gelatin emulsion gel (GEG)
Water	Solvent	92.85	35.51	26.10	33.88	25.26	21.00
Agar	Gelling	7.15	2.72	2.00	–	–	–
Pectin	agent	–	–	–	3.76	2.8	–
Gelatin		–	–	–	–	–	8.60
Citrem	Emulsifier	–	–	1.50	–	–	–
Xylitol	Sweeteners	–	38.64	28.40	38.10	28.4	28.40
Sorbitol		–	19.05	14.00	18.78	14	14.00
MA	Buffer	–	1.36	1.00	2.01	1.5	1.00
TCD	salts	–	2.72	2.00	2.68	2	2.00
Ascorbic acid		–	–	–	0.47	0.35	–
CCT	Calcium salt	–	–	–	0.32	0.24	–
Lecithin	Emulsifier	–	–	–	–	0.45	–
Corn oil	Oil	–	–	25.00	–	25.00	25.00

a similar way to AWO, with the exception of first adding TCD to the solution when the temperature was reduced to 65 °C. After TCD was fully dissolved, citrem was added to the mixture and mixed for 15 min with magnetic stirring. Afterwards, MA was added and mixed for 10 min. The water phase was degassed as described above. The temperature of the mixture was reduced to 50 °C and preheated corn oil at this temperature was included in the mixture. The water phase and the oil phase were homogenized at 50 °C using T18 digital ultra-turrax® (IKA®, USA) at speed 9.8k rpm for 8 min.

Pectin emulsion gel without oil (PWO), *i.e.*, pectin gel with sugar alcohols and buffer salts, was prepared by first dry mixing pectin and sorbitol, and then adding water and mixing with magnetic stirring at 75 °C for 25 min. Afterwards, xylitol, TCD, MA, ascorbic acid and CCT were dry mixed and gradually added to the mixture with a total mixing time of 20 min. The water phase was degassed as described above. Pectin emulsion gel (PEG) was prepared similarly to PWO. After degassing, the temperature was reduced to 60 °C, preheated lecithin was added, and mixed for 20 min. Preheated corn oil was added and homogenized at 60 °C with 9.8k rpm for 8 min.

Gelatin emulsion gel (GEG) was prepared by mixing gelatin with water for 30 min with magnetic stirring at 60 °C. Afterwards, sorbitol, xylitol, TCD and MA were added and mixed for 10 min after each addition. Preheated corn oil at 60 °C was added to the aqueous phase and homogenized at ambient temperature at 9.8k rpm speed for 5 min. The emulsion gel was degassed as described above.

The composition of the gels is given in Table 1. For AG, AWO and AEG, the ratio of agar to water was kept constant at 0.77. For PWO and PEG, pectin to water ratio was kept constant at 0.11.

2.3. Rheological characterization

Small amplitude oscillatory shear (SAOS) measurements on the gels were performed with a rheometer (Malvern Kinexus ultra+, Westborough, United States). The upper geometry was serrated PP40X SW1648 SS for all agar gels, whereas the lower plate was serrated PLS40X S1586 SS for AG and KNX0127, curved sandblasted 50 mm for AWO and AEG. For PWO, PEG and GEG, the lower plate was PL61 ST S2579 SS, and the upper geometry was CP4/40 40 mm diameter 4° cone angle. A solvent trap was used for AG, PEG and GEG to prevent evaporation during the measurement. The protocol given in Baydin et al. (2022) was followed. Instrument calibration (zero gap) was performed prior to analysis. After gel preparation, approximately 2 g of gel was placed on the Peltier temperature controlled lower plate, which was heated up to 60 °C. The rheometer was operated in 0.1% shear strain controlled mode and the frequency was set to 1 Hz. The chosen strain was confirmed to be within the linear viscoelastic region through a strain sweep performed on AG (one of the most brittle gels tested in this study) between 0.001 and 100% strain (Supplementary Figure 1). The viscoelastic properties of the sample were obtained by using a temperature gradient of 2 °C/min, with a start and end temperature at 60 °C and a holding time of 15 min at 20 °C for PEG and GEG. For AG, AEG and AWO, the end temperature was 90 °C and oscillation continued for 10 min at 90 °C. The results were analyzed using rSpace for Kinexus software (version 1.75) and Microsoft Excel (version 2201). The gelling temperature (T_g) and melting temperature (T_m) of the samples were estimated as the temperature at which the phase angle corresponded to 45° in the cooling and heating process, respectively. The maximum storage modulus (G'_{max}, Pa) was determined as the highest measurement point during setting at 20 °C.

2.4. Texture profile analyses

Texture properties of the gels were analyzed with TA.XT plusC Texture Analyser (Stable Micro Systems Ltd., UK). Upon preparation, the gels were cast using cylindrical molds of standard dimensions (19.6 mm height, 8 mm diameter). The gels were set at ambient temperature for 24 h prior to analysis. Single compression analysis and standard texture profile analysis (TPA) were performed at ambient temperature, using a 5 kg load cell for AG, AWO, AEG, PEG and PWO, and a 30 kg load cell for GEG and 75% strain TPA test of AG. P/35 35 mm diameter cylinder aluminum probe supplied by Stable Micro Systems Ltd. was used for both single compression and TPA analyses. For the 75% strain single compression, pre-test and post-test speeds were 2 mm/s, while the test speed was 0.1 mm/s and the trigger force was 5 g. Strain height was measured automatically during compression. Max force (g) and strain at failure (%) data was obtained from the fraction moment of the gels. Young's modulus (N/m^2) was calculated from the following equation where gradient (N/m) was calculated by the ratio of force at 2% and 3% strain:

$$Young's\ modulus\ \left(N/m^2\right) = \frac{Gradient\ (N/m) \times\ height\ of\ the\ gel\ (m)}{Area\ of\ the\ gel\ \left(m^2\right)}$$

Area of the gel was the contact area of the gel with the probe which was calculated from the surface area of the uniform cylinder molds.

The texture profile analysis (TPA) was carried out with 20%, 30% and 75% strain to mimic different components of mastication. Pre-test, test, and post-test speeds were 1 mm/s, and the trigger force was 5 g. Strain height was measured automatically during compression. Hardness, adhesiveness, resilience, cohesiveness, springiness, and chewiness parameters were calculated from the TPA data which were analyzed with the Exponent connect software. Gumminess parameter was excluded from the analysis since it is comparable to chewiness. Gumminess is valid for semi-solid materials, whereas chewiness is applicable for solids (Bourne, 2002).

Table 2
Compositions of control gels for *in vitro* lipolysis. All ingredients are given as weight percentage (w/w).

Ingredient	Agar emulsion gel control (AC)	Pectin emulsion gel control (PC)	Gelatin emulsion gel control (GC)
Water	52.60	50.71	46.00
Agar	2.00	–	–
Pectin	–	2.80	–
Gelatin	–	–	8.60
Xylitol	28.40	28.40	28.40
Sorbitol	14.00	14.00	14.00
MA	1.00	1.50	1.00
TCD	2.00	2.00	2.00
Ascorbic acid	–	0.35	–
CCT	–	0.24	–

2.5. Syneresis measurements

Syneresis measurements were based on weight loss of the gels upon freezing at −30 °C and thawing at ambient temperature (approximately 22 °C). The gel was weighed and sealed with an airtight foil, *i.e.*, aluminum blister. After freezing for 12 h and thawing for 4 h at ambient temperature, the gel was weighed again and the difference in gel weight was normalized to percentage loss.

2.6. Water activity measurements

The water activity (a_w) of the gels was measured with HygroPalm HC2-AW (Rotronic, Switzerland) at ambient temperature. The sample was placed into the measurement chamber and the water activity was recorded after 15 min.

2.7. Droplet size analyses

Droplet sizes of the emulsion gels were measured with the Mastersizer 3000 Hydro MV (Malvern, UK). After AEG and PEG were prepared, approximately 2 g of sample was dissolved in water at 50 °C. For GEG, the solvent was 0.1 M HCl. Solutions were added to the water cell drop by drop until an obscuration rate of 5–18% was obtained. The dispersant refractive index was set to 1.330 and 1.470, for water and corn oil, respectively. Particle absorption index was 0.010 for all emulsion gels. The data collected from the detectors was analyzed by the Mastersizer software. The software provided average droplet size parameters ($D[4,3]$ volume mean diameter and $D[3,2]$ surface mean diameter) of five measurements, as well as the droplet size distribution of each emulsion gel.

2.8. In vitro *lipolysis*

In vitro lipolysis was performed either as gastrointestinal (gastric lipolysis followed by intestinal lipolysis) stage or intestinal stage only. Digestion in the gastric stage was not monitored. Therefore, the gastric stage should be considered as a pretreatment before the intestinal stage. In addition to emulsion gels (GEG, PEG, and AEG) control gels of each emulsion gel (GC, PC and AC) were tested with gastrointestinal *in vitro* lipolysis setup. Control gels were prepared as described in Section 2.2 for the emulsion gels apart from not containing oil or emulsifier (Table 2). To mimic mastication, the polysaccharide gels were pushed through a metal mesh with pore size 1.7 mm (Endecott, England), whereas gelatin gels were dissected with a scalpel into similar size pieces as the polysaccharide gels. The *in vitro* lipolysis protocol previously reported by Dille and Draget, (2021) was followed with modifications.

In vitro lipolysis of the gels was performed at 37 °C. For the gastric stage, 1.5 g of gel was mixed with a NaCl solution. Rabbit gastric extract was added, and the pH was quickly and manually reduced to 3. The pH combination electrode was A 1622 M DI (SI Analytics, Germany) coupled to the titrator 7000-M1/20, TitroLine (SI Analytics, Germany). The simulated gastric fluid (SGF) was kept at constant stirring for 1 h. The final volume of the SGF was 10 ml, and the concentrations of NaCl and gastric lipase were 80 mM and 4 mg/ml, respectively. Afterwards, the contents of the SGF were transferred to a beaker containing simulated intestinal fluid (SIF).

The SIF was formed by adding 450 mg bile extract porcine (Sigma, USA, lot#SLCC9272) and water into the beaker and stirring the contents until the bile extract was fully dissolved. 12.5 ml salt mix solution (40 mM $CaCl_2$/536 mM NaCl/dH_2O) was added to the beaker and kept in magnetic stirring for 10 min. The pH was adjusted to 7 by manually adding droplets of 1 mM and 0.1 mM NaOH. Pancreatin from porcine pancreas (Sigma, USA, lot#SLBZ5739) and lipase from porcine pancreas (Sigma, USA, lot#SLBZ7254) were dissolved in water in separate Eppendorf tubes through vortex mixing. The titrator was set to maintain the pH at 7 by adding droplets of 0.1 mM NaOH. The pH-stat method was initiated after simultaneously adding 1 ml of each enzyme mixture into the beaker. The final concentrations of the SIF components were 9 mg/ml bile extract porcine, 10 mM $CaCl_2$, 134 mM NaCl, 1.2 mg/ml pancreatin and 1.2 mg/ml lipase. The final volume of the SIF was 50 ml. The SIF was kept at constant stirring for 1 h while the data from the pH-stat method were recorded through the titrator.

For tests with only the intestinal *in vitro* lipolysis, the salt mix contained 40 mM $CaCl_2$ and 600 mM NaCl to reach a final concentration of 10 mM $CaCl_2$ and 134 mM NaCl in SIF without the SGF. For the *in vitro* lipolysis curves, the total lipolysis was assumed to start when pH initially reached pH 7, *i.e.*, the data points until pH 7 were removed and the total NaOH consumption was calculated after this point. The initial rate of the *in vitro* lipolysis reaction was calculated from the slope of the total lipolysis curve between 4 and 80 s.

2.9. Microscopy

Photomicrographs of the emulsion gels were taken after 1 h of gastric lipolysis using inverted light microscope Axio Observer Z1 (Carl Zeiss Microscopy GmbH, Germany). A small amount of SGF was transferred with a Pasteur pipette on the microscope slide, covered with a cover slip and examined using the microscope. The ZEISS ZEN pro digital imaging software (Version 2.3) was used to photograph the samples.

2.10. Statistical analyses

All statistical analyses were performed using IBM® SPSS® Statistics software version 28.0.1.0 (142). One-way analysis of variance (ANOVA) with post-hoc Tukey's honest significant difference (HSD) test was performed. Statistically significant differences were reported if $p < 0.05$.

3. Results and discussion

The emulsion gels were characterized and compared using water activity (a_w), syneresis, SAOS, large scale deformation, and droplet size measurements. To study the impact of oil in the polysaccharide gels, AEG and PEG were also compared to their "without oil" (WO) versions, AWO and PWO, respectively. In addition, AWO and AEG were compared

Table 3
Water activity (a_w) and syneresis of the gels presented as% water loss. For a_w and syneresis measurements, three and 6–9 replicates per each sample were analyzed, respectively. For a_w, the standard deviation was smaller than 0.01 for each gel. The abbreviations used for the gels are given in Table 1. *This result was due to minor adhesion of the gel to the aluminum blister. Different letters in superscript indicate statistically significant differences between gels. For both a_w and syneresis $p < 0.001$ between different gels.

Gel	a_w	Average syneresis (%)
AG	0.94 ^{d}	5.6 ± 0.7 ^{d}
AWO	0.77 ^{bc}	4.7 ± 0.6 ^{c}
AEG	0.77 ^{c}	3.2 ± 0.2 ^{b}
PWO	0.74 ^{a}	1.5 ± 0.3 ^{a}
PEG	0.76 ^{b}	1.7 ± 0.4 ^{a}
GEG	0.73 ^{a}	1.0 ± 0.5 *

to a pure agar gel (AG). In the WO gels, the aqueous phase was identical to the emulsion gels; for any ingredient in the WO gel, solute/water ratio is equal to its corresponding emulsion gel. Because of this, similar a_w was observed for emulsion gels and their WO versions. The a_w of different emulsion gels were proportional to the water they contain in the formulation (Table 1, Table 3). As expected, the a_w of AG was much higher than AWO and AEG, highlighting the importance of co-solutes to decrease the a_w of food systems below 0.85 to prevent microbial growth as recommended by the U.S. Food and Drug Administration (2018).

Syneresis, *i.e.*, expulsion of water from a gel, is a common phenomenon for agar gels (Armisen & Gaiatas, 2009). As expected, AG showed the highest syneresis (Table 3). The addition of sugar alcohols to AG significantly lowered syneresis for AWO, in accordance with lower a_w. The inclusion of oil in the formulation further reduced syneresis for AEG significantly. In a previous study with alginate emulsion gels, this was suggested to be due to oil droplets acting as barriers for water transport (Lević et al., 2015; Lin et al., 2020a). It is known that increasing agar concentrations result in lower syneresis (Banerjee & Bhattacharya, 2011). The current data suggest that the inclusion of sugar alcohols and oil also contribute to reducing syneresis due to a reduced amount of water as well as a greatly reduced a_w (free water) in the system. Pectin gels are known to exhibit syneresis during storage as a result of aging (Rao, Van Buren & Cooley, 1993). Although syneresis was lower for both PEG and PWO compared to agar gels, the inclusion of oil did not reduce the syneresis of the pectin gel (Table 3).

GEG was not included in the statistical tests for syneresis (Table 3). In this system, syneresis was considered negligible since no sweating was observed. This is in accordance with literature (Mizrahi, 2010). In general, syneresis in food gels is an undesirable phenomenon since it may impact the quality and stability of the product (Banerjee & Bhattacharya, 2011; Mizrahi, 2010). However, for adhesive gels which exhibit stickiness to their packaging material, a controlled amount of syneresis may promote lubrication due to surface liquid (Saha & Bhattacharya, 2010). During the syneresis measurements no stickiness was observed with agar or pectin gels.

3.1. Small amplitude oscillatory shear measurements

Although agar/water ratio was kept constant for the agar gels (AG, AWO, and AEG), it should be noted that AG had a much higher overall concentration of agar in the total formulation. In addition, water-soluble polyols increase the total volume of the water phase, resulting in a lower agar/water phase ratio for AWO and AEG. Consequently, AG had the significantly highest G'_{max} among agar gels, followed by AWO and AEG, although without significant differences (Table 4). In accordance with the literature, inactive fillers decrease gel strength compared to an oil-free gel (Dickinson & Chen, 1999; Dille, Haug & Draget, 2021b). Similarly, PWO had a higher G'_{max} than PEG, although the difference was not statistically significant. Among emulsion gels, AEG had the highest and PEG had the lowest G'_{max}, respectively.

Table 4
G'_{max}, T_m, and T_g of emulsion gels, their "without oil" versions and a pure agar gel. The results are presented as the average of three independent replicates ± standard deviation. The abbreviations used for the gels are given in Table 1. N/A indicates that data could not be obtained since the phase angle has not reached 45° with the current temperature gradient. The results were obtained with a temperature gradient of 2 °C/min and are expected to vary between different temperature gradients. Different letters in superscript indicate statistically significant differences between gels. For all three parameters $p < 0.001$ between different gels.

	G'_{max} (kPa)	T_m °C	T_g °C
AG	145.9 ± 33.0 ^{d}	N/A	50.7 ± 6.8 ^{b}
AWO	73.9 ± 1.7 ^{c}	87.4 ± 1.1 ^{c}	39.1 ± 1.1 ^{a}
AEG	44.1 ± 7.8 ^{bc}	90.1 ± 0.2 ^{c}	40.2 ± 1.3 ^{a}
PWO	6.1 ± 0.2 ^{ab}	50.5 ± 2.2 ^{b}	36.3 ± 1.0 ^{a}
PEG	3.2 ± 0.3 ^{a}	54.2 ± 2.7 ^{b}	38.3 ± 1.8 ^{a}
GEG	16.8 ± 1.7 ^{ab}	45.3 ± 1.3 ^{a}	37.0 ± 0.5 ^{a}

Although the T_m of AG could not be obtained, it is expected to be above 90 °C, due to high agar concentration (Lahrech, Safouane & Peyrellasse, 2005). The T_m and T_g of AWO and AEG were similar with a slightly higher T_m for AEG. The T_m of AEG (90 °C) was significantly higher than PEG (54 °C) and GEG (45 °C) which were also significantly different (Table 4). A high T_m may be advantageous for the storage and textural stability of polysaccharide-based gel products in warm climates. The stability of gelatin gels is known to be compromised at high storage temperatures due to the acid hydrolysis of gelatin (Baydin et al., 2022; Van den Bosch & Gielens, 2003). However, the high T_m of AEG may present challenges during commercial production since it would require critical temperature control to ensure the quality of the final product. Although agar gels have a reversible gelation process without significant compromising the gelling properties (Imeson, 2009), AEG had a lower G'_{max} after re-melting the gel (data not shown). For industrial production, PEG may be a more manufacturable formulation alternative to GEG since it has relatively similar T_m and T_g values to GEG and it did not exhibit large decreases in G'_{max} after re-melting (data not shown). It should be noted that PEG may pose challenges for industrial applications due to the complexity and sensitivity of its formulation to, *e.g.*, pH and calcium concentration (Burey, Bhandari, Rutgers, Halley & Torley, 2009; Zeeb et al., 2021).

3.2. Texture characteristics of the gels

Consumer acceptance of emulsion gels for oral delivery highly depend on their mechanical properties which describe their response to deformation, such as mastication, *i.e.*, chewing (Aguayo-Mendoza et al., 2020). Texture characteristics of the gels were analyzed with a single compression and TPA tests with three maximum strains. The average curves of single compression with 75% strain are shown in Fig. 1A. The parameters of this test demonstrated that AG had the significantly highest Young's modulus among all gels, indicating high stiffness/rigidity. Although the inclusion of sugar alcohols (AWO) significantly decreased the Young's modulus, it significantly increased the maximum force and strain at failure of the agar gel (Table 5). The lower Young's modulus of AWO is due to a lower overall agar concentration in the formulation compared to AG. Strain at failure coincides with the sensory deformability of the material (Koç et al., 2019). As discussed in Section 3.1, AG had a much higher overall concentration of agar in the total formulation compared to AWO and AEG. The stiffness order of agar gels (AG > AWO > AEG) obtained with single compression were in accordance with the G'_{max} values obtained by SAOS measurements (Table 4).

Both agar and pectin WO gels had significantly higher Young's modulus, maximum force and strain at failure, compared to their corresponding emulsion gels, although the difference was not significant for the Young's modulus of PWO and PEG (Table 5). As expected, the inclusion

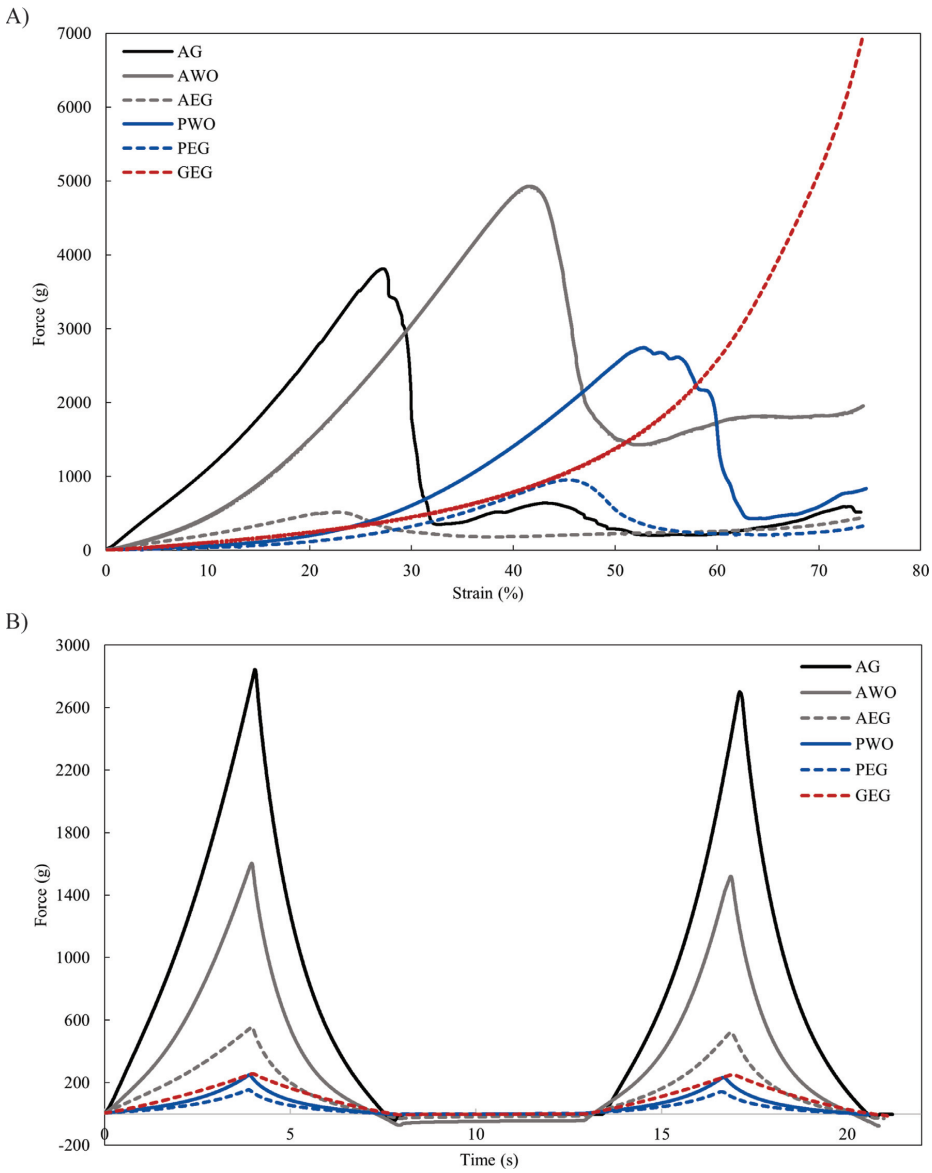

Fig. 1. A) Large scale deformation of the gels with texture analyzer. 75% strain single compression was applied. The curves are averages of 6–10 replicates (obtained from a single batch). B) Texture profile analysis of the gels with 20% strain. Each curve represents the average of 7–10 gels (obtained from a single batch). The abbreviations and compositions of the gels are given in Table 1.

of oil in the agar and pectin formulations resulted in a more brittle and weaker texture as the oil droplets behave as inactive fillers (Kim et al., 1997, 1999; Zhang et al., 2022). The emulsified oil droplets may provide an inactive filler effect with the continuous phase which results in a weaker polysaccharide gel network and provide possible failure zones for crack propagation (Dickinson, 2012; Dille, Draget & Hattrem, 2015; Sala, van de Velde, Stuart & van Aken, 2007). AEG showed significantly higher brittleness than PEG (Table 5).

GEG had the significantly highest maximum force among all gels, and it did not fail at 75% strain, pointing to the ductile texture of the gel (Fig. 1A, Table 5). The polysaccharide emulsion gels had a significantly lower maximum force, and they were significantly more brittle than GEG (Table 5). The brittleness of polysaccharide gels has been attributed to their high chain stiffness, as opposed to gelatin with long and flexible cross-links (Van Vliet & Walstra, 2017). Compared to AEG, although significantly different, PEG had strain at failure and maximum

Table 5
Parameters of large scale deformation (75% strain single compression) of the gels with texture analyzer. The results are given as the average of 6–9 replicates (obtained from a single batch) ± standard deviation. The abbreviations of the gels are given in Table 1. Different letters in superscript indicate statistically significant differences between gels. For all three parameters $p < 0.001$ between different gels.

	Young's modulus (kN/m^2)	Max force (g)	Strain at failure (%)
AG	552.4 ± 31.7 ^{e}	3439 ± 906 ^{b}	25 ± 5 ^{a}
AWO	182.0 ± 24.7 ^{d}	4993 ± 227 ^{c}	42 ± 1 ^{b}
AEG	95.0 ± 1.9 ^{c}	523 ± 39 ^{a}	22 ± 1 ^{a}
PWO	21.5 ± 1.1 ^{ab}	3299 ± 573 ^{b}	56 ± 4 ^{c}
PEG	12.6 ± 0.3 ^{a}	975 ± 100 ^{a}	46 ± 1 ^{b}
GEG	46.2 ± 3.7 ^{b}	7349 ± 150 ^{d}	> 75 ^{d}

force values closer to GEG, suggesting a more similar sensory perception. Young's modulus and strain at failure parameters were significantly different between the emulsion gels ($p < 0.001$), pointing to their different deformation characteristics.

Gelatin gels have a unique chewy texture that is familiar to consumers of gelatin-based jelly desserts, gummies, and confectionery (Schrieber & Gareis, 2007). This texture is difficult to be mimicked with polysaccharides since polysaccharide gels typically do not have a similar elastic/chewy texture (Haug, Draget & Smidsrød, 2004; Karim & Bhat, 2008). The texture of the gels was analyzed with standard TPA tests at three different strains (20%, 30%, and 75%) which represent a variety of strain at failure values obtained from the single compression test. These strain values also represent different anatomical components of mastication such as the tongue, hard palate and teeth (Arai & Yamada, 1993). It has been shown that different degrees of compression, *i.e.*, maximum strain, impact TPA parameters (Bourne & Comstock, 1981). Standard TPA parameters (hardness, adhesiveness, cohesiveness, resilience, springiness, and gumminess) were obtained for each gel at three different strains.

Fig. 1B shows TPA curves of the gels with 20% strain, which is below the strain at failure for all gels. The textural properties of the gels are shown in Table 6. Similar to the Young's modulus values, hardness of the gels was in the following order for agar: AG > AWO > AEG, and PWO > PEG for pectin gels with significant differences, pointing out to weaker gel structure due to the inactive filler effect (Farjami & Madadlou, 2019; Koç et al., 2019). Hardness, gumminess, and adhesiveness of the emulsion gels were in the following order: AEG > GEG > PEG, and AWO showed the significantly highest adhesiveness among all gels. Resilience, springiness, and cohesiveness parameters describe the structural integrity of the gel after the first compression. The springiness value was close to 100, and cohesiveness value was one for all gels since none of the gels fractured at this strain. The significantly lower resilience of the polysaccharide emulsion gels, compared to gelatin emulsion gel, indicated some structural damage, especially for AEG which had a strain at failure closer to 20% strain than PEG (Table 6). All parameters of the 20% strain TPA test (excluding cohesiveness) were significantly different between the gels ($p < 0.001$).

The only fractured gel during 30% strain TPA test was AEG, and therefore, its cohesiveness value was zero unlike the other gels which had a cohesiveness value of one (Supplementary Table 1). Consequently, it also had significantly lower resilience and springiness than the other gels ($p < 0.001$). Compared to 20% strain TPA test, the springiness and resilience of all gels decreased, pointing to structural damage. Similar to the 20% strain TPA test, polysaccharide emulsion gels had significantly lower hardness and gumminess compared to their WO gels, indicating the negative impact of the inactive filler effect on these parameters. The hardness and adhesiveness of the emulsion gels followed the same order as 20% strain TPA (Table 6, Supplementary Table 1). The 30% strain TPA curves of the gels are shown in Supplementary Figure 2. All parameters of the 30% strain TPA test (excluding cohesiveness) were significantly different between the gels ($p < 0.001$).

As expected, all polysaccharide gels fractured at 75% strain TPA test (reflected by the cohesiveness values). At this strain, GEG had a significantly higher hardness and gumminess than the polysaccharide emulsion gels (Supplementary Figure 3). Although it did not fracture at 75% strain, GEG's low resilience indicated structural damage. The hardness of GEG increased with increasing maximum strain of the TPA (Table 6, Supplementary Table 1, Supplementary Table 2). At 75% strain, the gel is close to its elastic limit, resisting deformation to a larger extent (Mazumder, Roopa & Bhattacharya, 2007). The presence of oil significantly decreased the hardness and gumminess of the polysaccharide gels, whereas it had little effect on the resilience, cohesiveness, and springiness. Although not significantly different, PEG had a slightly higher hardness than AEG. All parameters of the 75% strain TPA test (excluding cohesiveness) showed statistically significant differences between the gels ($p < 0.001$).

Both single compression and 75% strain TPA test compressed the gels to the same extent. However, maximum force and fracture strain results of the single compression test (0.1 mm/s) were lower than those obtained from the hardness results of the TPA test (1 mm/s). This may be due to the compression speed differences of the two tests. A slower compression rate gives the gel more time to relax and dissipate the applied force through friction between structural components of the gel at higher deformation speeds (Pons & Fiszman, 1996; Sala, Van Vliet, Stuart, Van Aken & Van de Velde, 2009). In previous studies, compression rates of 2–3 mm/s were suggested to be more physiologically relevant to mastication (Pons & Fiszman, 1996; Rosenthal, 2010).

The texture analyses indicated that at high strains (75%), the addition of sugar alcohols to the pure agar gel resulted in higher hardness, whereas lower strains (20 and 30%) showed the opposite. The addition of sugar alcohols resulted in a less brittle texture and further addition of oil to the agar formulation led to a lower hardness at all strains. Structure maintained in the gel after deformation was as followed: AEG > AWO > AG, suggesting that the addition of polyols and oil to a pure agar gel resulted in a texture with higher structural integrity (Supplementary Figure 4). AEG had a harder, firmer, and more brittle texture than GEG. The addition of oil weakened the pectin gels at all strains. PEG had lower hardness than both AEG and GEG. However, it was less brittle and had higher resilience, cohesiveness, and springiness than AEG, potentially making its mouthfeel more similar to GEG.

Table 6
Textural properties of the gels obtained with 20% strain TPA test. The results are given as the average of (7–10) gels (obtained from a single batch) ± standard deviation. The abbreviations and compositions of the gels are given in Table 1. Cohesiveness value for all the gels was 1 ± 0. Different letters in superscript indicate statistically significant differences between gels.

Gel	Hardness (g)	Adhesiveness (g·sec)	Resilence (%)	Springiness (%)	Gumminess
AG	2853 ± 175 ^{e}	−30 ± 24 ^{c}	65 ± 1 ^{c}	91 ± 1 ^{a}	2404 ± 160 ^{e}
AWO	1614 ± 53 ^{d}	−271 ± 83 ^{a}	61 ± 1 ^{b}	93 ± 1 ^{b}	1366 ± 50 ^{d}
AEG	558 ± 25 ^{c}	−101 ± 16 ^{b}	56 ± 1 ^{a}	96 ± 1 ^{c}	459 ± 20 ^{c}
PWO	251 ± 4 ^{b}	−9 ± 4 ^{c}	78 ± 1 ^{e}	99 ± 1 ^{d}	227 ± 4 ^{b}
PEG	155 ± 4 ^{a}	4 ± 2 ^{c}	76 ± 1 ^{d}	100 ± 0 ^{d}	139 ± 4 ^{a}
GEG	255 ± 11 ^{b}	−14 ± 7 ^{c}	92 ± 2 ^{f}	99 ± 1 ^{d}	249 ± 11 ^{b}

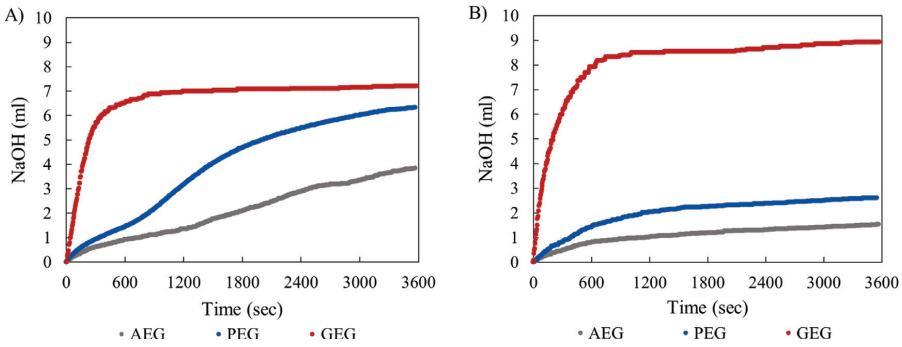

Fig. 2. Total NaOH consumption of agar (AEG), pectin (PEG) and gelatin (GEG) emulsion gels during *in vitro* lipolysis in A) gastrointestinal and B) intestinal stage. The curves show total NaOH consumption without subtracting the consumption of the corresponding control gels.

Table 7
The D[4, 3] and D[3, 2] droplet size averages of agar (AEG), pectin (PEG) and gelatin (GEG) emulsion gels. The D[4, 3] and D[3, 2] droplet size averages are given as the average of three replicate gels with five measurements ± standard deviation. The results of the *in vitro* lipolysis are given as the average of two experiments ± standard deviation. Different letters in superscript indicate statistically significant differences between groups.

	Droplet size averages		Initial lipolysis rate (μmol FFA/*sec*)	
	D[4, 3] (μm)	D[3, 2] (μm)	Gastrointestinal	Intestinal
AEG	13.2 ± 1.5 ^{a}	3.0 ± 0.8 ^{a}	2.75 ± 0.07 ^{a}	2.45 ± 0.07 ^{a}
PEG	31.5 ± 5.1 ^{b}	9.7 ± 1.7 ^{b}	4.30 ± 0.14 ^{a}	3.50 ± 1.13 ^{a}
GEG	1.0 ± 0.2 ^{c}	0.6 ± 0.0 ^{c}	24.85 ± 7.57 ^{b}	35.15 ± 6.29 ^{b}

Texture analyses demonstrated different characteristics of agar, pectin, and gelatin emulsion gels. These differences will impact the sensory perception, aroma release profile, and dissolution time of the emulsion filled gels (Sala et al., 2008). A lower pH of PEG (pH 4) may result in faster flavor release than AEG or GEG (pH 4.5) (Hansson, Andersson, Leufven & Pehrson, 2001). One of the most commonly used biopolymers in vegan confectionery is pectin, which may have a familiar texture for vegan consumers (Šeremet et al., 2020). In Asia, AEG may have higher consumer acceptance since agar has traditionally been used as a food ingredient for centuries (Sousa et al., 2021).

3.3. *Droplet size and* in vitro *lipolysis of emulsion gels*

The droplet size of an emulsion is known to influence the digestion, uptake, and bioavailability of TAGs (Dille, Baydin, Kristiansen & Draget, 2021a). Especially the surface mean diameter, *i.e.*, D[3, 2], is important since a lower average provides a larger surface area accessible for the digestive enzymes. For both D[3, 2] and D[4, 3], GEG had the smallest droplet size, whereas PEG had the largest (Table 7). Both droplet size averages were significantly different between emulsion gels ($p < 0.001$).

The lipolysis of the emulsion gels was studied through *in vitro* experiments. To evaluate the decrease in pH due to the experimental setup, gastrointestinal lipolysis was performed with a "blank" sample (water instead of a gel), as a background control. The total NaOH consumption was 0.40 ± 0.11 ml ($n = 3$). This indicates the background lipolysis reaction occurring, possibly due to the presence of lipid impurities in the bile extract or the lipolysis of pancreatin (Larsen, Sassene & Müllertz, 2011). During gastrointestinal lipolysis, the net NaOH consumption is calculated as the total consumption of the control gel (AC, PC, and GC) subtracted from the total consumption of the corresponding emulsion gel (AEG, PEG, and GEG) (Section 2.8). The control gels were only subjected to gastrointestinal lipolysis, *i.e.*, control gels have not been tested in the intestinal stage. In the gastrointestinal stage, GEG had the highest net consumption with 6.09 ± 0.44 ml, followed by PEG with 5.22 ± 0.44 ml and lastly AEG with 3.18 ± 0.34 ml (Fig. 2A). The total NaOH consumption in the intestinal stage was 8.93 ± 0.67 ml for GEG, followed by 2.61 ± 0.13 ml for PEG and 1.53 ± 0.01 ml for AEG (Fig. 2B). The reason for GEG having a higher total NaOH consumption in the intestinal stage compared to the gastrointestinal stage may be because of the free fatty acids released from the triacylglycerols (TAGs) in the SGF becoming deprotonated as the pH is raised to 7 before the intestinal stage. Therefore, less TAGs would be available in the intestinal stage to be hydrolyzed and release fatty acids that are detected by the pH-stat method since some of the TAGs were already hydrolyzed to diacylglycerols or monoacylglycerols in the gastric stage. The lipolysis curves of the gels are shown in Fig. 2.

In both gastrointestinal lipolysis and intestinal lipolysis, the titration curves of polysaccharide emulsion gels have not reached a plateau during 1 hour of titration. A higher total NaOH consumption might have been recorded for the gels if the intestinal titration was prolonged. A longer duration of titration could, however, have given more physiologically relevant results since the gastrointestinal transit time in humans is longer than 1 hour (Read, Al-Janabi, Holgate, Barber & Edwards, 1986; Worsøe et al., 2011).

The initial lipolysis rate of the emulsion gels followed the same order for both gastrointestinal and intestinal lipolysis: GEG > PEG > AEG. In both gastrointestinal ($p < 0.025$) and intestinal ($p < 0.005$) lipolysis, the initial rate of different emulsion gels was significantly different with GEG being different from the polysaccharide emulsion gels (Table 7). Larger oil droplets are lipolyzed at a slower rate compared to small oil droplets which provide a larger surface area for the digestive enzymes to adsorb to (Dille et al., 2021a). Although AEG had a smaller droplet size than PEG, its initial lipolysis rate was slower. The initial rate of AEG and PEG were lower in intestinal stage, compared to their relative rate in the gastrointestinal stage. However, the opposite was observed for GEG, with 1.4x faster initial lipolysis in the intestinal stage.

The photomicrographs of the emulsion gels after the gastric stage showed coalescence of oil droplets in AEG (Fig. 3A). Similar observations have been reported for citrem stabilized emulsions in the gastric stage at pH 1.3 and 3, and this was suggested to be due to the lowered emulsifying capacity of citrem with the loss of negative charges at lower pH values (Lamothe, Desroches & Britten, 2019, 2020; Sørensen et al., 2008). The photomicrographs of PEG indicated flocculated oil droplets (Fig. 3B). At low pH values, emulsions stabilized by soybean lecithin have been shown to destabilize due to reduced emulsifying capacity of the emulsifier (Comas, Wagner & Tomás, 2006; Lin & Wright, 2018; Lin, Wang, Li & Wright, 2014). Simultaneously, pectin may contribute to the stabilization of the emulsion, keeping the emulsion droplets in an aggregated but not coalesced state, because of bridging flocculation (Ngouémazong, Christiaens, Shpigelman, Van Loey & Hendrickx, 2015).

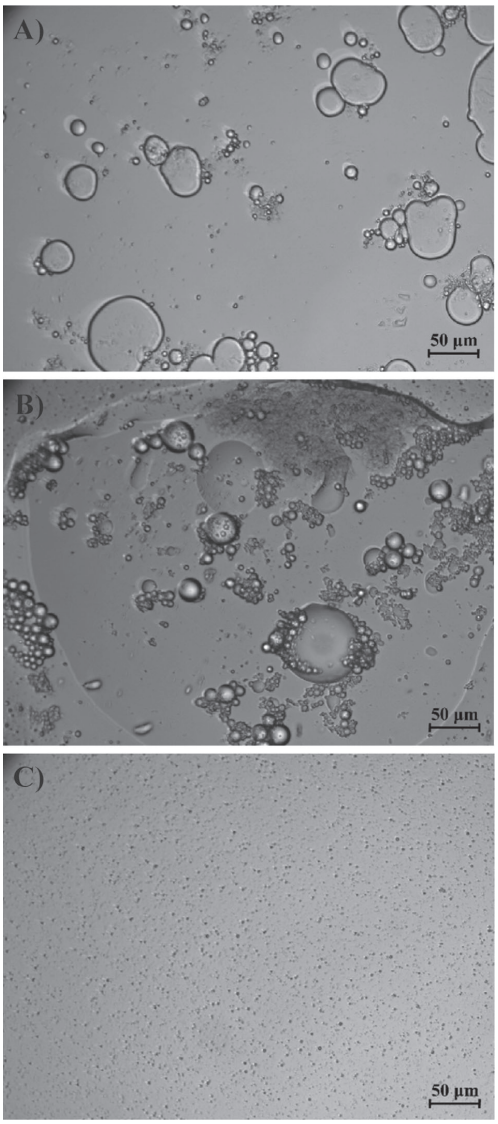

Fig. 3. Photomicrographs of A) agar, B) pectin, and C) gelatin emulsion gel after the gastric stage. 20x objective was used.

Coalescence and flocculation of oil droplets reduce the available substrate area for digestive enzyme adsorption and reduce the total extent and rate of lipolysis (Li et al., 2011; McClements, 2018). The lipolysis curves of gastrointestinal lipolysis of AEG and PEG show an increase at approximately 1000 s which was not observed for the intestinal lipolysis without a gastric pretreatment (Fig. 2). For PEG, this increase in NaOH consumption may be due to the increase of available substrate for the lipases in the intestinal stage at pH 7 which may disperse the flocs and create more available surface. It should also be noted that calcium ions are absent in the gastric stage. Calcium helps with the precipitation of FFAs, removing them from the surface of the oil droplets, and increasing the accessibility of the surface to the digestive enzymes (Minekus et al., 2014). Furthermore, the absence of calcium in SGF may mediate dissolution of the pectin network since calcium binds to pectin and facilitates its gelling (Fraeye, Duvetter, Doungla, Van Loey & Hendrickx, 2010). For AEG, the increase may be due to the gel remaining in the lipolysis medium for a longer period (two hours in the gastrointestinal stage, instead of one hour in the intestinal stage), with coalesced oil droplets being lipolyzed further, exposing a larger substrate area for the lipases. This would also promote shrinking of oil droplets due to lipase activity, further resulting in droplet release from the gel network.

It should be noted that *in vitro* lipolysis does not directly indicate the outcome of lipolysis *in vivo*. More complex *in vitro* lipolysis experiments with greater physiological relevance have been developed (Brodkorb et al., 2019), and the limitations of *in vitro* lipolysis experiments are well known (Ghorbani & Abedinzade, 2013). The current simplified setup serves a means to compare the lipolysis potential of emulsion gels, prepared with different biopolymers which contain the same amount of oil. Lower initial lipolysis rate, as well as lower total NaOH consumption for AEG and PEG in both gastrointestinal and intestinal stages, compared to GEG, point to limitations for the digestibility of oils in polysaccharide emulsion gels. These limitations may suggest potential lower absorption of polysaccharide emulsion gels *in vivo*, and consequently result in lower bioavailability. However, in the human body, shear forces in the stomach may result in a higher extent of mechanical deformation of the polysaccharide gels, resulting in smaller gel particles exposing a larger surface area available to lipases.

4. Conclusions

Emulsion gels using agar and pectin were prepared, characterized, and compared to a traditional gelatin emulsion gel. The higher melting temperature of agar emulsion gel may be advantageous in warm climates for enhanced stability of the final product, while it may pose difficulties in processability. The pectin emulsion gel had a more similar melting/gelling temperature to gelatin emulsion gel. The addition of sugar alcohols and oil to a pure agar gel decreased hardness and brittleness, potentially resulting in a more pleasant mouth-feel. Pectin emulsion gel had a softer texture than the agar emulsion gel, and a more similar texture to the gelatin emulsion gel. In the future, the textural properties of the emulsion gels should be further studied through a sensory panel using *e.g.*, quantitative descriptive analysis. *In vitro* lipolysis studies showed a lower extent of lipolysis and slower initial lipolysis rate with polysaccharide emulsion gels compared to the gelatin emulsion gel. Absorption and bioavailability of these three systems should be further studied using an *in vivo* model, and preferably in humans. The polysaccharide emulsion gels can also be used for other applications, as food gels or for the oral delivery of pharmaceuticals.

Ethical statement

The research presented does not involve any animal or human study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

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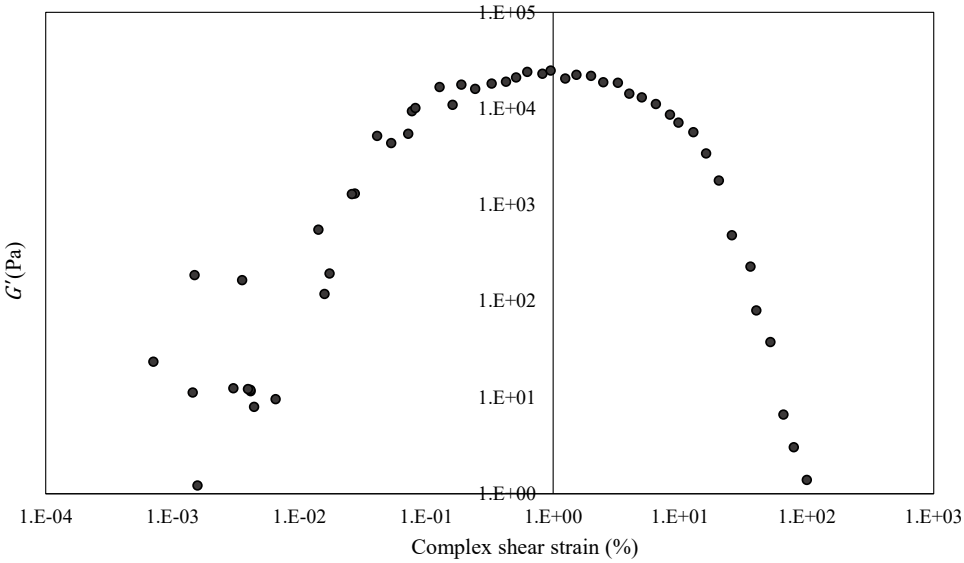
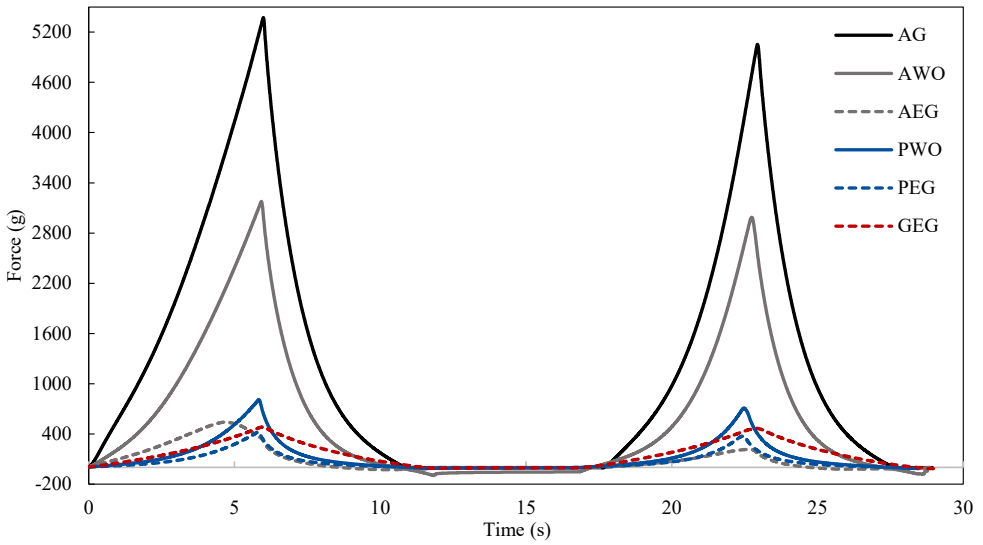
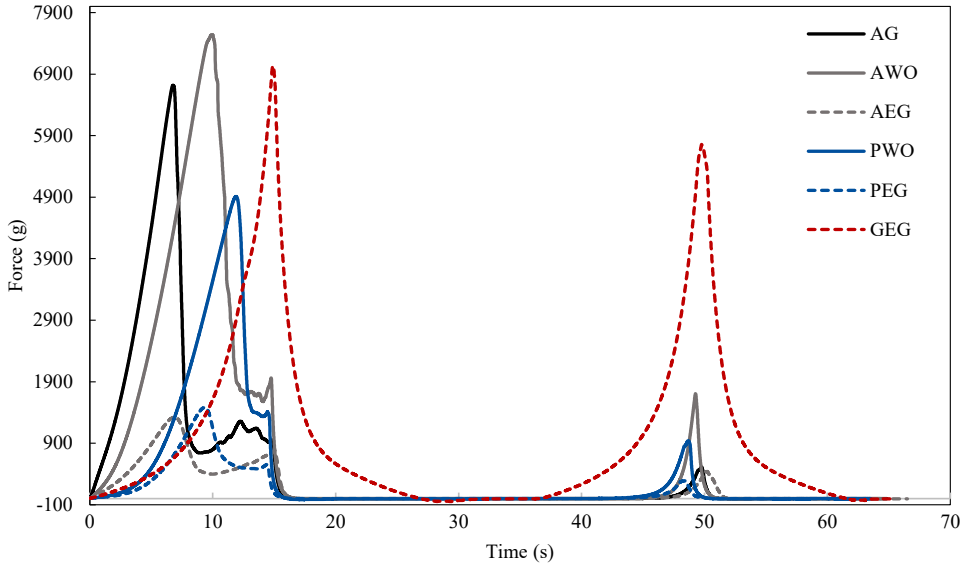
Supplementary Data



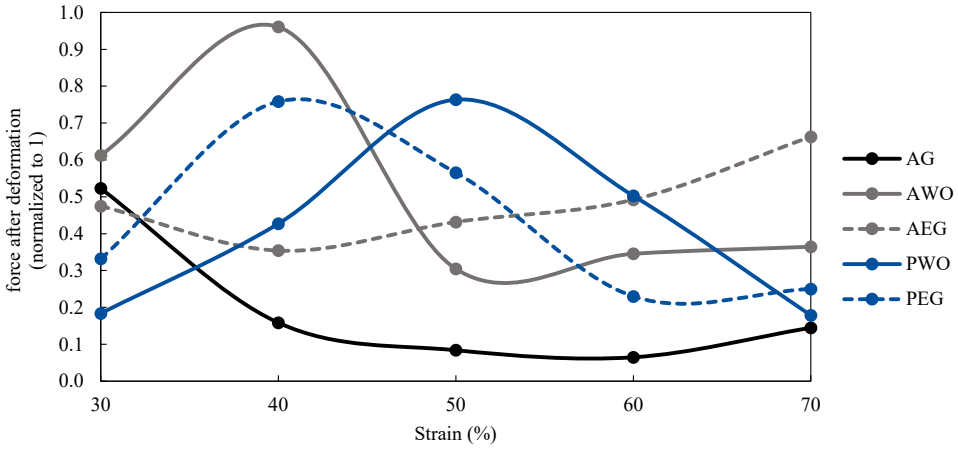
Supplementary Figure 1: Strain sweep of 7.15% agar gel (AG).



Supplementary Figure 2: Texture profile analysis of the gels with 30% strain. Each curve represents the average of 7-10 gels (obtained from a single batch). The abbreviations and compositions of the gels are given in Table 1.



Supplementary Figure 3: Texture profile analysis of the gels with 75% strain. Each curve represents the average of 7-10 gels (obtained from a single batch). The abbreviations and compositions of the gels are given in Table 1.



Supplementary Figure 4: Force after gel deformation at strains from 30 to 70%. The maximum force at single compression was regarded as 1 and force after deformation was normalized to 1. The abbreviations and compositions of the gels are given in Table 1.

Supplementary Table 1: Textural properties of the gels obtained with 30% strain TPA test. The results are given as the average of (6-10) gels (obtained from a single batch) ± standard deviation. The abbreviations and compositions of the gels are given in Table 1. Cohesiveness value for all the gels was 1 ± 0 except for AEG (0 ± 0). Different letters in superscript indicate statistically significant differences between gels.

	Hardness (g)	Adhesiveness (g.sec)	Resilence (%)	Springiness (%)	Gumminess
AG	5379 ± 118 ^{e}	-25 ± 10 ^{b}	50 ± 0 ^{c}	88 ± 0 ^{ab}	3963 ± 138 ^{f}
AWO	3190 ± 86 ^{d}	-365 ± 149 ^{a}	46 ± 1 ^{b}	92 ± 2 ^{bc}	2370 ± 56 ^{e}
AEG	524 ± 52 ^{b}	-93 ± 35 ^{b}	14 ± 2 ^{a}	85 ± 7 ^{a}	159 ± 32 ^{a}
PWO	816 ± 16 ^{c}	-46 ± 7 ^{b}	55 ± 1 ^{d}	95 ± 1 ^{cd}	646 ± 8 ^{d}
PEG	427 ± 9 ^{a}	-13 ± 4 ^{b}	56 ± 1 ^{d}	98 ± 1 ^{d}	341 ± 5 ^{b}
GEG	490 ± 23 ^{ab}	-35 ± 14 ^{b}	87 ± 1 ^{e}	98 ± 1 ^{d}	479 ± 21 ^{c}

Supplementary Table 2: Textural properties of the gels obtained with 75% strain TPA test. The results are given as the average of (8-13) gels (obtained from a single batch) ± standard deviation. The abbreviations and compositions of the gels are given in Table 1. Cohesiveness value for all the gels was 0 ± 0 except for GEG (1 ± 0). Different letters in superscript indicate statistically significant differences between gels.

	Hardness (g)	Adhesiveness (g.sec)	Resilence (%)	Springiness (%)	Gumminess
AG	6946 ± 610 ^{c}	-6 ± 6 ^{b}	1 ± 1 ^{a}	38 ± 8 ^{a}	159 ± 86 ^{a}
AWO	7839 ± 583 ^{d}	-20 ± 31 ^{b}	1 ± 1 ^{a}	40 ± 11 ^{a}	347 ± 136 ^{b}
AEG	1320 ± 61 ^{a}	-92 ± 39 ^{b}	3 ± 1 ^{b}	52 ± 20 ^{ab}	114 ± 28 ^{a}
PWO	5006 ± 398 ^{b}	-82 ± 47 ^{ab}	1 ± 0 ^{a}	58 ± 12 ^{b}	327 ± 48 ^{b}
PEG	1494 ± 86 ^{a}	-13 ± 18 ^{ab}	2 ± 0 ^{b}	49 ± 12 ^{ab}	104 ± 15 ^{a}
GEG	7430 ± 244 ^{cd}	-159 ± 130 ^{a}	50 ± 0 ^{c}	89 ± 2 ^{c}	5842 ± 181 ^{c}

Paper 3

Paper 4. The impact of emulsion droplet size on *in vitro* lipolysis rate and *in vivo* plasma uptake kinetics of triglycerides and vitamin D_3 in rats

M. J. Dille, T. Baydin, K. A. Kristiansen, and K. I. Draget (2021). "The impact of emulsion droplet size on *in vitro* lipolysis rate and *in vivo* plasma uptake kinetics of triglycerides and vitamin D_3 in rats". In: *Food & Function* 12, pp. 3219-3232. DOI: 10.1039/d0fo03386c.

The main idea of the paper was proposed by M. J. Dille with contributions by K. I. Draget. The experimental work was conducted by M. J. Dille, with contributions of T. Baydin with *in vivo* experiments. The mass spectrometry analyses were carried out by K. A. Kristiansen. The paper was structured and written by M. J. Dille, with editing by all co-authors.

Paper 5. A comparative study of different plant proteins as potential emulsifiers in agar containing emulsions

Baydin, T., M. N. Hattrem, and K. I. Draget (2022). "A comparative study of different plant proteins as potential emulsifiers in agar containing emulsions". Manuscript in preparation.

The main idea of the paper was proposed by T. Baydin with contributions by all co-authors. The experimental work was caried out by T. Baydin. The paper was structured and written by T. Baydin, with editing by all co-authors.

This paper is in preparation for submission and is therefore not included.

Patent application. Chewable plant-based agar gel containing an oil-in-water emulsion and a plant-based macromolecular surfactant for the oral delivery of nutraceuticals and pharmaceuticals

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(71) Applicant: **VITUX GROUP AS** [NO/NO]; Brynsveien 11-13, 0667 Oslo (NO).

(71) Applicant *(for MG only)*: **GOLDING, Louise** [GB/GB]; St Bride's House, 10 Salisbury Square, London Greater London EC4Y 8JD (GB).

(72) Inventors: **DILLE, Morten J**; c/o Vitux Group AS, Brynsveien 11-13, 0667 Oslo (NO). **BAYDIN, Tuna**; c/o Vitux Group AS, Brynsveien 11-13, 0667 Oslo (NO). **HATTREM, Magnus**; c/o Vitux Group AS, Brynsveien 11-13, 0667 Oslo (NO). **DRAGET, Kurt Ingar**; c/o Vitux Group AS, Brynsveien 11-13, 0667 Oslo (NO).

(74) Agent: **DEHNS**; St Bride's House, 10 Salisbury Square, London Greater London EC4Y 8JD (GB).

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(54) Title: COMPOSITIONS

(57) Abstract: The invention relates to compositions for oral administration which are provided in the form of gelled oil-in-water emulsions, to methods for their preparation and to their use as pharmaceuticals and nutraceuticals. In particular, it relates to orally administrable, gelled oil-in-water emulsions which are self-supporting, viscoelastic solids having a gelled aqueous phase comprising a gelling agent which is agar, and wherein said emulsion is stabilised by a surfactant which is a plant-based protein, plant-based polysaccharide or derivative thereof.

WO 2022/219358 A1

PATENT

Compositions

Field of the invention

The present invention generally relates to compositions for oral administration which are provided in the form of gelled oil-in-water emulsions, to methods for their preparation and to their use as pharmaceuticals and nutraceuticals. The compositions are soft, yet chewable, and can be provided in a unit dosage form which is easy to swallow. More specifically, the invention relates to oral compositions which are acceptable to patients and consumers that wish to abstain from the consumption of animal by-products, for example those that follow a vegetarian diet or who are vegan. It also relates to oral compositions that are acceptable to pescetarians.

Background of the invention

Soft chewable dosage forms are an alternative to traditional oral administration forms such as tablets, capsules, elixirs and suspensions. They are easier to swallow than tablets and capsules and are particularly suitable for the pediatric and elderly population as well as those that suffer from dysphagia. Such dosage forms are a popular choice for dietary supplements which contain vitamins and/or minerals (so-called “nutraceuticals”), and are also suitable for the delivery of active pharmaceutical ingredients (APIs). Active components (nutraceutical or pharmaceutical) may be present in the form of lipids in gelled oil-in-water emulsions, as dispersed particulates or dissolved in the oil or aqueous phase of such emulsions.

A range of gelling agents can be used in the preparation of soft chewable dosage forms, such as gelled oil-in-water emulsions, however gelatin is by far the most widely used due to its availability, ease of use and its sol-gel transition temperature. Gelatin is produced by partial hydrolysis of collagen found in the skin, bones and connective tissue of animals and is most commonly derived from pork, bovine and fish sources. The sol-gel transition temperature of a gelatin generally corresponds to the body temperature of the animal from which it is obtained. Gelatins from mammalian sources therefore have transition temperatures which are similar to

human body temperature, resulting in gels which are solid at room temperature but which melt in the mouth once ingested. Gelatin-based dosage forms thus provide a pleasant 'melt-in-the mouth' texture or "mouthfeel". Gelatin also provides fast and consistent dissolution kinetics of a dosage unit in the gastrointestinal tract which can be beneficial to promote rapid uptake of any active components.

Gelatin has significant surface active properties which allows it to act as an emulsifier as well as a gelling agent. This makes it a particularly good choice for use as a gelling agent to produce oil-in-water emulsions which are chewable. Gelatin-based emulsions typically experience an "active filler effect" in which the droplets of oil interact strongly with the surrounding gel network and are generally referred to as "active fillers". When the oil droplets are sufficiently small, this interaction between the gel network and the oil droplets increases the storage modulus of the gelled emulsion compared to an oil-free gel, i.e. the gel alone. In contrast, oil droplets which are distributed throughout a gel with little or no interaction with the gel network are known as "inactive fillers" and result in a modulus for the gelled emulsion which is lower than that of the gel alone. When oil droplets of a gelled emulsion are present as "inactive fillers", the emulsion may not be stable over time. That can lead to destabilization of the emulsion and 'sweating' of oil.

Despite the many advantageous properties of gelatin for use in the production of soft chewable dosage forms, its animal origin makes it unacceptable to many patients and consumers due to their religious beliefs or dietary choices. As an animal by-product, gelatin is not acceptable to vegans for example.

Gelling agents that are not of animal origin and which have previously been proposed for use in the production of soft chewable dosage forms, such as gelled oil-in-water emulsions, include non-proteinaceous materials such as alginates, carrageenans and pectins. However, the gelling properties of these materials can be difficult to control due to the need for their complexation with metal ions, temperature change and/or pH adjustment to produce the desired 'gel'. This is not ideal in the context of a dosage form which is to be manufactured on a commercial scale.

An alternative gelling agent which is widely used in food and other non-food applications is agar. Agar is extracted from marine red algae and comprises a polysaccharide containing galactose sub-units. It is a thermosetting polymer which gels at about 30-45°C. Agar melts at about 85-90°C and once melted it retains a liquid state until cooled to 40°C. Due to its large hysteresis between gelling and melting temperatures it has the potential for use in the large scale production of dosage units formed from gelled oil-in-water emulsions. However, unlike gelatin which produces soft, flexible gels that can withstand a high degree of compression before they break, agar-based gels are hard and brittle. Whereas a gelatin-based gel might withstand up to 70-90% compression before it breaks, for example, an agar-based gel will typically fragment under a deformation of as little as 20%. This severely restricts its use in the production of any dosage unit that needs to be soft and chewable and have a pleasant mouthfeel.

Unlike gels based on gelatin, agar-based gels are also prone to syneresis, i.e. spontaneous release of water from the gel on ageing. Gels are a 3D network of polymers which cross-link with one another trapping water within their structure. If the polymer network is not disturbed, the water remains in place. Over time, however, the polymers which form the gel may contract or alter their conformation causing water to be expelled and shrinkage of the gel. Oozing of water out of the gel is known as “syneresis” and this must be minimised in any oral dosage unit due to the need for it to have an adequate shelf-life, i.e. it should remain stable over an extended period of time. One of the ways in which the problem of syneresis of agar gels has traditionally been addressed is by increasing the agar concentration. However, that can lead to a harder, more solid and more brittle gel which is undesirable when seeking to provide a soft, chewable dosage form.

There is thus a continuing need for alternative soft, yet chewable, compositions for the oral delivery of pharmaceuticals and/or nutraceuticals that are suitable for vegetarians, pescetarians and vegans. In particular, there is a need for such compositions that can provide an acceptable alternative to conventional gelatin-based oil-in-water emulsions in terms of their “chew” and mouthfeel characteristics. Such compositions should be capable of manufacture on a commercial scale and have adequate stability (i.e. shelf-life) for use as pharmaceutical and/or nutraceutical products.

The present invention addresses at least some of these needs.

Summary of the invention

The Applicant now proposes gelled oil-in-water emulsions that are acceptable to patients and consumers that are vegetarian, pescetarian or vegan, in particular to those that follow a vegetarian or vegan diet. The emulsions employ agar as a gelling agent and are stabilised using certain plant-based surfactants. Specifically, the emulsions are stabilised by at least one surface-active protein or polysaccharide derived from a plant or a derivative thereof. When used to stabilise agar-based, gelled oil-in-water emulsions the Applicant has found that these high molecular weight (i.e. "macromolecular"), plant-based surfactants are advantageous compared to low molecular weight surfactants. In particular, they have found that these macromolecular surfactants provide gelled emulsions that are stable and which possess desirable rheology characteristics for the oral delivery of active agents in a soft, yet chewable, dosage form.

In one aspect the invention provides an orally administrable, gelled oil-in-water emulsion which is a self-supporting, viscoelastic solid having a gelled aqueous phase comprising a gelling agent which is agar, and wherein said emulsion is stabilised by a surfactant which is a plant-based protein, a plant-based polysaccharide, or a derivative thereof.

In another aspect the invention provides a method for the preparation of a gelled oil-in-water emulsion as herein described, said method comprising the steps of: forming an oil phase which comprises one or more physiologically tolerable lipids; forming an aqueous phase comprising a gelling agent which is agar; combining said oil phase and said aqueous phase to form an oil-in-water emulsion in the presence of a surfactant which is a plant-based protein, plant-based polysaccharide, or a derivative thereof; and allowing said emulsion to gel.

In a further aspect the invention provides a gelled oil-in-water emulsion as herein described for oral use as a medicament or for oral use in therapy.

In another aspect the invention provides a gelled oil-in-water emulsion as herein described which contains at least one pharmaceutically active component for oral use in the treatment of a condition responsive to said pharmaceutically active component.

In another aspect the invention provides the use of a pharmaceutically active component in the manufacture of a medicament for oral use in the treatment of a condition responsive to said pharmaceutically active component, wherein said medicament is provided in the form of a gelled oil-in-water emulsion as herein described.

In another aspect the invention provides a method of treatment of a human or non-human animal subject (e.g. a patient) to combat a condition responsive to a pharmaceutically active agent, said method comprising the step of orally administering to said subject a pharmaceutically effective amount of said agent in the form of a gelled oil-in-water emulsion as herein described.

In another aspect the invention provides the use of a gelled oil-in-water emulsion as herein described as a nutraceutical.

Detailed description of the invention

Definitions

The term “gel” refers to a form of matter that is intermediate between a solid and a liquid. The formation of a “gel” will typically involve the association or cross-linking of polymer chains to form a three-dimensional network that traps or immobilises solvent (e.g. water) within it to form a sufficiently rigid structure that is resistant to flow at ambient temperature, i.e. at a temperature below about 25°C, preferably below about 20°C. In rheological terms, a “gel” may be defined according to its storage modulus (or “elastic modulus”), G’, which represents the elastic nature (energy storage) of a material, and its loss modulus (or “viscous modulus”), G”, which represents the viscous nature (energy loss) of a material. Their ratio, tan δ (equal to G”/G’), also referred to as the “loss tangent”, provides a measure of how much the stress and strain are out of phase with one another.

A material which is "viscoelastic" is characterised by rheological properties which resemble, in part, the rheological behaviour of a viscous fluid and, also in part, that of an elastic solid.

The gelled oil-in-water emulsions according to the invention are "self-supporting, viscoelastic solids". This is intended to mean that they exhibit characteristics intermediate between those of a solid and a liquid, but have a dominant solid behaviour, i.e. they have rheological characteristics more similar to that of a solid than a liquid. A "solid dominant behaviour" cannot be diluted away (i.e. destroyed) by adding more solvent. In contrast, in the case of a weak (or entangled) gel lacking stable (i.e. long lived) intermolecular crosslinks, the entangled network structure of the gel can be removed by adding more solvent and can readily be destroyed even at very low shear rate/shear stress.

The gelled oil-in-water emulsions of the invention exhibit mechanical rigidity, yet in contrast to a solid they are deformable. Specifically, the gelled emulsions herein described have a storage modulus, G', which is greater than their loss modulus, G", (i.e. G' > G") over a wide frequency range, for example in the frequency range from 0.001 to 10 Hz when measured at ambient temperature (i.e. at a temperature in the range of 18°C to 25°C, e.g. at 20°C) and 0.1% strain. Storage modulus and loss modulus may be measured using known methods, for example using a Kinexus Ultra+ Rheometer applying a C 4/40 measuring geometry. Storage modulus and loss modulus values are not expected to differ when measured using other types of rheometer within the linear viscoelastic range.

More specifically, the gelled oil-in-water emulsions herein described will have the following properties: G' > G" over a frequency range of 0.001 to 10 Hz at 0.1% strain; and a storage modulus (G') at ambient temperature (i.e. at a temperature in the range of 18°C to 25°C, e.g. at 20°C) in the range from 10 to 200,000 Pa, preferably 100 to 100,000 Pa, more preferably 500 to 50,000 Pa.

Weak gels will typically have a loss tangent, tan δ > 0.1. For strong gels, or fully developed gels, G' >> G" and lower tan δ values (< 0.1) are observed. The gelled oil-in-water emulsions herein described would generally be considered "strong gels"

at ambient temperature, i.e. at a temperature in the range of 18°C to 25°C, e.g. at 20°C.

As used herein, the term "gelled" refers to the formation of a "gel". The term is used herein both in relation to the physical nature of the aqueous phase of the emulsion and that of the oil-in-water emulsion. As will be understood, the oil droplets act more or less like a solid when dispersed throughout the gelled aqueous phase of the oil-in-water emulsions which are the subject of the invention. The "gelled" nature of the aqueous phase is thus also a characteristic of the oil-in-water emulsion, i.e. it can also be considered "gelled" as described herein.

Unless otherwise defined, the term "liquid" as used herein refers to a substance which flows freely and which maintains a constant volume. It includes thickened liquids and viscous liquids which flow. A "liquid" has a loss modulus (G") which is greater than its storage modulus (G') and a loss tangent (tan δ) which is greater than 1.

As used herein, the term "surfactant" refers to a surface active compound or composition which is capable of reducing the interfacial tension between two immiscible liquids, e.g. at the interface between oil and water. Typically, a surfactant will be amphiphilic in nature and will comprise both hydrophobic and hydrophilic components. It may consist of a single component or may be a mixture of components. Where the surfactant is a mixture of components, the individual components will typically, though not necessarily, be similar in structure. To the extent that the surfactant for use in the invention is obtained from a natural product (i.e. from a plant or plant part), it will be understood that it will typically comprise a mixture of different components. The surfactant may be a naturally-occurring product obtained from a plant or part of a plant, or it may be a derivative thereof as described herein (i.e. it may be semi-synthetic).

As used herein, the term "fatty acid" refers to an un-branched or branched, preferably un-branched, hydrocarbon chain having a carboxylic acid (-COOH) group at one end, conventionally denoted the α (alpha) end. The hydrocarbon chain may be saturated or (mono- or poly-) unsaturated. By convention, the numbering of the carbon atoms starts from the α-end such that the carbon atom of

the carboxylic acid group is carbon atom number 1. The other end, which is usually a methyl (-CH_3) group, is conventionally denoted ω (omega) such that the terminal carbon atom is the ω-carbon. Any double bonds present may be cis- or trans- in configuration. The nomenclature "ω-x" is used to signify that a double bond is located on the xth carbon-carbon bond, counting from the terminal carbon (i.e. the ω-carbon) towards the carbonyl carbon.

By "physiologically tolerable" is meant any component which is suitable for administration to a human or non-human animal body, in particular which is suitable for oral administration.

By "pharmaceutical" is meant any product intended for a medical purpose, e.g. for treating or preventing any disease, condition or disorder of a human or non-human animal body, or for preventing its recurrence, or for reducing or eliminating the symptoms of any such disease, condition or disorder. The use and production of a product as a "pharmaceutical" will be closely regulated by a government agency. It may, but need not, be prescribed by a physician. For example, it may be available "over the counter", i.e. without a prescription.

"Treatment" or "treating" includes any therapeutic application that can benefit a human or non-human animal (e.g. a non-human mammal). Both human and veterinary treatments are within the scope of the present invention, although primarily the invention is aimed at the treatment of humans. Veterinary treatment includes the treatment of livestock and domestic animals (e.g. pets such as cats, dogs, rabbits, etc.). Treatment may be in respect of an existing disorder or it may be prophylactic.

In contrast to a pharmaceutical, a "nutraceutical" need not be the subject of regulatory approval. The term "nutraceutical" is used herein to refer to a product which is generally considered beneficial to maintain or augment the health and/or general well-being of a human or non-human animal subject. Such substances include, in particular, dietary supplements such as vitamins and minerals which are intended to augment the health of a subject (e.g. a human subject).

As will be understood, some substances may be considered both a "pharmaceutical" and a "nutraceutical". Categorization of a substance as one or the other, or indeed both, may vary in different countries depending on local regulations relating to medicinal products. It may also be dependent on the recommended daily dosage of any given substance. Higher daily doses of certain vitamins such as vitamin D, for example, may be regulated as a pharmaceutical whereas lower daily dosages may be considered nutraceutical.

By "a pharmaceutical composition" is meant a composition in any form suitable to be used for a pharmaceutical purpose.

By a "nutraceutical composition" is meant a composition in any form suitable to be used for a nutraceutical purpose.

A "pharmaceutically effective amount" relates to an amount that will lead to the desired pharmacological and/or therapeutic effect, i.e. an amount of the agent which is effective to achieve its intended pharmaceutical purpose. While individual patient needs may vary, determination of optimal ranges for effective amounts of any active agent is within the capability of those skilled in the art.

A "nutraceutically effective amount" relates to an amount that will lead to the desired nutraceutical effect, i.e. an amount of the agent which is effective to achieve its intended nutraceutical purpose. While the individual needs of a subject may vary, determination of optimal ranges for effective amounts of any active agent is within the capability of those skilled in the art.

The term "capsule" is used herein to refer to a unitary dosage form having a casing or coating (herein referred to as the "capsule shell") which encloses a gelled oil-in-water emulsion as herein defined.

As used herein, "water activity" is the partial vapour pressure of water in a composition at a specified temperature divided by the standard state partial vapour pressure of water at the same temperature. Water activity thus acts as a measure of the amount of free (i.e. unbound) water in a composition. Water activity may be

measured by methods known to those skilled in the art, for example by using a Rotronic Hygrolab instrument.

As used herein, an "animal by-product" is intended to refer to any product derived from, isolated from, or purified from one or more parts of an animal body (e.g. bone, skin, tissue, meat, cartilage, hoof, horn, etc.). It is also intended to refer to any composition preparing by processing an animal by-product, for example, derivatised, functionalised, or otherwise chemically or physically modified, animal by-products. As used herein, an "animal by-product" is not intended to include milk, eggs, or any compound or composition that is derived from, isolated from, or purified from animal milk or animal eggs. The term "animal by-product" does not include any synthetic material, or any material obtained from any plant, fungal, bacterial or algal source.

As used herein, the term "vegetarian diet" generally refers to a diet that lacks any meat and which also lacks any animal by-product as herein defined. A "vegetarian diet" may include animal milk and animal eggs and any products derived, isolated or purified therefrom. Such a diet may also be generally known as an "ovo-lactovegetarian" diet or "lacto-ovovegetarian" diet which, in addition to food from plants, includes milk, cheese, other dairy products and eggs. A "pescetarian diet" refers to a diet in which the only source of meat is fish and seafood. A "vegan diet" refers to a diet that is totally vegetarian and which includes only food from plants (e.g. fruit, vegetables, grains, legumes, seeds and nuts). Any reference herein to a product, substance, composition or formulation which is "suitable for" a given diet means that it would be acceptable for those that follow that particular diet. The terms "vegetarian", "pescetarian" and "vegan" are intended to refer to those who follow a vegetarian, pescetarian or vegan diet, respectively.

In a first aspect the invention provides an orally administrable, gelled oil-in-water emulsion which is a self-supporting, viscoelastic solid having a gelled aqueous phase comprising a gelling agent which is agar, and wherein said emulsion is stabilised by a surfactant which is a plant-based protein, a plant-based polysaccharide, or a derivative thereof.

The aqueous phase of the emulsion according to the invention comprises water and is gelled using agar as a gelling agent. The aqueous phase is also referred to herein as the "continuous phase" of the emulsion. The gelling agent may be a single type of gelling agent or it may be a mixture of different types of gelling agents. Where more than one gelling agent is used, at least one of the agents will be agar.

Agar is well known and used in the art, for example in food and other non-food applications. It is envisaged that any known type of agar may be used in the invention. As used herein, the term "agar" is intended to broadly define any product which contains a hydrocolloidal polysaccharide extracted from red seaweed, i.e. a seaweed of the family Rhodophyceae. The hydrocolloidal polysaccharide present in agar contains one or more polymers made up of subunits of galactose. Sources of agar include seaweeds belonging to the following genera: *Gelidium*, *Gracilaria*, *Pterocladia* and *Gelidiella*. *Gracilaria* genus is the major source of agar globally.

The nature of the agar and its properties (e.g. its gelling capacity) will vary depending on the species from which it is extracted and the extraction method used in its production, but it is envisaged that any known agar may find use in the invention. Agars obtained from *Gracilaria* species are typically more sulfated and therefore have a lower gelling capacity. However, their gelling properties may be enhanced by alkaline hydrolysis of the seaweed material prior to extraction. This converts the L-galactose 6-sulfate units into 3,6-anhydro-L-galactose residues which are considered to be responsible for the gelling properties of the polymer. Alternatively, pre- and/or post-extraction, agars may be subjected to enzyme treatment to remove sulfate groups.

Agars are linear polysaccharides made up of alternating β (1,3)- and α (1,4)-linked galactopyranose residues. A substantial part of the α-galactose residues may exist as the 3,6-anhydride derivative. The (1,3)-linked residue is the D-enantiomer, while the (1,4)-linked residue is the L-enantiomer. Natural chemical modifications of these structures by acidic groups (namely sulfate, uronate and pyruvate) as well as by non-ionic methoxy groups have been identified. Early studies suggested that agar consisted of two main fractions: a neutral fraction termed "agarose" having high gelling ability, and a charged fraction called "agaropectin" having a lower

gelling ability. More recent studies have shown that agar is a complex mixture of polysaccharides ranging from essentially neutral to charged galactan molecules. The term "agarose" refers to the neutral polysaccharide with high gelling ability made up of repeating disaccharide units of agarobiose, i.e. 4-O-(β-D-galactopyranosyl)-3,6-anhydro-α-L-galactopyranose. The polysaccharide with repeating disaccharide units of 4-O-(β-D-galactopyranosyl)-α-L-galactopyranose in which the anyhydride bridge is absent is called "agaran". Alkaline treatment of agar removes the sulfate ester on the C6 of the 4-linked galactose units with formation of the corresponding 3,6-anhydride form. This treatment is widely used in industrial agar extraction from *Gracilaria* sp. to improve its gelling properties. A more detailed overview of agar can be found, for example, in Chapter 24 of the Handbook of Hydrocolloids (Sousa et al., 2021), the entire content of which is incorporated herein by reference.

Agar is globally permitted in food products by Food Safety Authorities, including the European Food Safety Authority (EFSA) as a food additive (E-406) and the Food and Drug Administration (FDA). Agar is supplied as a powder having high solubility in water, for example at least 85% (at 80°C). Its gel strength may vary but will typically be in the range from about 700 to about 1100 g/cm^2 (measured in respect of a 1.5 wt.% concentration in water at 20°C). The gelling point of agar is typically in the range from 30 to 45°C (measured at a 1.5 wt.% concentration in water at 20°C). The melting point of agar may, for example, range from 80 to 95°C (measured at a 1.5 wt.% concentration in water at 20°C). Agar having a gelling point in the range from about 35 to about 45°C (measured at a 1.5 wt.% concentration in water at 20°C) and/or a melting point of from about 80 to 95°C, e.g. from about 85 to 92°C (measured at a 1.5 wt.% concentration in water at 20°C) is particularly preferred for use in the invention.

Agar for use in the invention can be obtained from various commercial sources. Non-limiting examples of agars which may be used include Gelagar HDR 800 (from B. & V. srl, Italy), and Qsol™ High Solubility Agar and Qsol Agar (from Hispanagar, Spain).

The aqueous phase of the gelled oil-in-water emulsions according to the invention can comprise agar as the sole gelling agent, or it may comprise additional non-agar

gelling agents. Where other gelling agents are present, these may be selected from other gelling agents known in the art. Consistent with the intended "vegetarian" or "vegan" nature of any of the products defined herein, any additional gelling agent should not be any animal by-product. For example, mammalian gelatin will not be present. Preferably, gelatin from any source (including fish gelatin) will not be present.

The gelling agent or combination of gelling agents will be present in the aqueous phase in an amount suitable to provide the desired degree of gelling as herein described. The amount will vary to some extent dependent on the precise nature of the gelling agent(s) (for example, the type of agar which is employed) and/or other components of the aqueous phase, but a suitable amount may readily be determined by those skilled in the art. Where a gelling agent other than agar is also employed, an appropriate amount may readily be selected by those skilled in the art. The amount of agar may be adjusted accordingly.

In one set of embodiments, agar may be present in the aqueous phase at a concentration of about 0.1 to about 7.5 wt.%, preferably about 0.25 to about 5 wt.%, particularly about 0.3 to about 3.5 wt.%, e.g. about 0.5 to about 3 wt.% (i.e. based on the weight of the aqueous phase). For example, it may be present at a concentration of 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0 wt.% (based on the weight of the aqueous phase). The concentration of agar based on the overall weight of the composition may range from about 0.1 to about 5 wt.%, preferably from about 0.15 to about 4.5 wt.%, more preferably from about 0.2 to about 4 wt.%, e.g. from about 0.25 to about 3.5 wt.%, or from about 0.25 to about 3 wt.%. For example, it may be present at a concentration of 0.25, 0.50, 0.75, 1.0, 1.25, 1.50, 1.75, 2.0, 2.25, 2.5, 2.75 or 3.0 wt.% (based on the overall weight of the composition).

The gelled emulsions herein described are stabilised by a surfactant which is a plant-based protein or polysaccharide. As used herein, the term "plant-based" is intended to refer to a material that is derived (e.g. extracted) from a plant or any part of a plant, such as the fruit or seed of a plant. Such materials include derivatives of any naturally-occurring component of a plant or plant part, for example derivatives obtained by chemical modification. The plant-based surfactant

for use in the invention may thus be a natural product, or it may be a semi-synthetic product.

The surfactant for use in the invention is capable of stabilising a gelled oil-in-water emulsion as herein described. In order to perform this function, it will be understood that the surfactant should be sufficiently soluble in the aqueous phase of the emulsion under the conditions used to produce the emulsion. Due to the nature of some of the surfactants proposed for use in the invention, specifically those which contain plant proteins, solubility should take into account the pH of the aqueous phase. In one embodiment, the surfactant will have a solubility of at least about 5 mg/ml in an aqueous solution at a pH of 4.5 when measured at a temperature of about 50°C and at a pressure of about 1 atm.

The surfactant for use in the invention will be one which is suitable for use in on oral pharmaceutical or a food product. It may, for example, be any surfactant which is acceptable for use in a food product, i.e. a food grade protein, polysaccharide or any derivative thereof which is suitable for human consumption. Typically it will be a surfactant which has been approved for use as a food additive by a food-related administration (e.g. the European Food Safety Authority, or the US Food and Drug Administration). Surfactants having an E-number and which are therefore permitted for use as food additives within the European Union are particularly suitable for use in the invention.

The plant-based surfactant for use in the invention comprises a plant protein, a plant polysaccharide, or any derivative or combination thereof. Derivatives include products obtained by chemical and/or physical modification of plant proteins, plant polysaccharides and mixtures thereof. Chemical modification may include, for example, functionalisation to introduce one or more functional groups, or hydrolysis to reduce the molecular weight of the material. Functionalisation is particularly suitable since it can be employed to adjust the hydrophobic/hydrophilic characteristics of the product. Suitable functional groups and methods for their introduction are well known in the art. Non-limiting examples of functional groups include, for example, aliphatic groups, carboxyl, amine and amide groups. Functionalisation may also involve reaction with another compound to form a conjugate, for example reaction with a glycol such as propylene glycol ("PG").

Physical methods may include, but are not limited to, ultra-purification for example to tailor the molecular weight distribution of the material.

Plant protein derivatives which are suitable for use in the invention include, for example, hydrolysed proteins. Derivatives of plant polysaccharides for use in the invention include, for example, polysaccharides that are hydrophobically modified to impart the desired surface active properties and/or water-solubility.

The plant protein and plant polysaccharide surfactants for use in the invention are high in molecular weight and will generally be considered "macromolecular". In one embodiment, the plant-based surfactant will have a weight average molecular weight, Mw, which is greater than or equal to about 10 kDa, for example which is greater than or equal to about 15 kDa, 20 kDa or 25 kDa. Typically, the plant-based surfactants for use in the invention will have a weight average molecular weight ranging from about 10 to about 500 kDa, for example from about 20 to about 450 kDa, or from about 25 to about 450 kDa, or from about 30 to about 450 kDa, from about 40 to about 450 kDa, from about 50 to about 450 kDa, from about 60 to about 450 kDa, or from 70 to about 450 kDa, or from 80 to about 450 kDa. In another set of embodiments, the surfactant for use in the invention may have a weight average molecular weight which ranges from about 10 to about 80 kDa, preferably from about 20 to about 70 kDa, e.g. from about 30 to about 70 kDa. Methods for the measurement of molecular weight are well known in the art. That typically used for measuring the molecular weight of any protein, for example, is SEC-MALLS (Size Exclusion Chromatography - Multiple Angle Laser Light Scattering).

Plant proteins and their derivatives having surface active properties and which are suitable for use in the invention are well known in the art. Plant proteins are typically supplied in two major forms: isolate and concentrate. Unless otherwise specified, any reference herein to "a protein" includes the protein in the form of the isolate and concentrate. Concentrates may include fat, carbohydrates and bioactive compounds, for example. Isolates are processed to remove the fat and carbohydrates and, in some cases, may also be lower in any bioactive compounds.

Plants in the legume family (Fabaceae or Leguminosae) are a significant source of proteins known for use in food products and may be used in the invention. Typically such proteins are obtained from the fruit or seed of the plant. The family Fabaceae includes, for example, Glycine max (soy bean), Phaseolus sp. (genus of beans), Pisum sativum (pea), Cicer arietinum (chickpeas), and Arachis hypogaea (peanut). Examples of legumes from which protein materials for use in the invention may be derived include, but are not limited to, peas, beans, chickpeas, lentils, soy beans (also known as soya beans) and peanuts. Other plant-based proteins which may be used in the invention include those obtained from rice, sunflower, potato, and chia, for example.

Proteins in legumes include water-soluble albumins, and salt-soluble globulin storage proteins (7S vicilin and/or 11S legumin fractions) (see, for example, Boye et al., “Pulse proteins: Processing, characterization, functional properties and applications in food and feed" - Food Research International 43(2): 414-431, 2010, the entire content of which is incorporated herein by reference). These globular proteins consist of polymorphic subunits bound together by primarily non-specific hydrophobic interactions; vicilin is a trimer, while legumin is a hexamer (see, for example, Schwenke "Reflections about the functional potential of legume proteins A Review" - Food / Nahrung 45(6): 377-381, 2001, the entire content of which is incorporated herein by reference). Legume proteins are relatively high in beta-sheet structures compared to cereal or animal protein, imparting a high structural flexibility. This aids emulsion stabilization as the proteins undergo significant conformational changes upon adsorbing to emulsion droplets, exposing hydrophobic residues to the oil phase and forming a highly stable interfacial layer (see, for example, Tang et al., "A comparative study of physicochemical and conformational properties in three vicilins from Phaseolus legumes: Implications for the structure–function relationship" - Food Hydrocolloids 25(3): 315-324, 2011; and Sharif et al., "Current progress in the utilization of native and modified legume proteins as emulsifiers and encapsulants - A review" - Food Hydrocolloids 76: 2-16, 2018, the entire contents of which are incorporated herein by reference).

Particularly suitable for use in the invention are proteins obtained from peas or beans, including isolates and concentrates of such proteins. Those from soy bean and faba bean are particularly suitable.

Pea and bean protein isolates are highly refined or purified forms of pea and bean protein. Pea protein isolate typically has a minimum protein content of about 80% (dry basis), whereas bean protein isolate may have a minimum protein content of about 65%, sometimes as high as 90% (dry basis). Pea protein can be obtained from a variety of species of pea. Bean protein can be obtained from a variety of species of bean including, but not limited to, faba bean and soy bean. Soy protein isolates are a highly refined or purified form of soy protein with a minimum protein content of about 90% (dry basis). Soy protein isolates are made from defatted soy flour from which most of the non-protein components, such as fats and carbohydrates, have been removed. Pea and bean proteins, including isolates and concentrates, are suitable for vegetarian and vegan diets. Commercial sources of pea protein include Nutralys and Hill Pharma. Commercial sources of bean protein, for example, faba bean protein include Vestkorn and Hill Pharma. Commercial sources of soy protein include PHH (Supro 590).

Plant polysaccharides and their derivatives having surface active properties and that are suitable for use as surfactants in the invention are well known in the art. These include, for example, celluloses, starches, alginates and derivatives thereof. In many cases, these materials will be chemically modified to impart the required surface active properties and/or to provide the desired degree of water solubility.

In one embodiment, the polysaccharide for use in the invention will be a hydrophobically-modified polysaccharide. A “hydrophobically-modified polysaccharide” means a polysaccharide that incorporates one or more hydrophobic groups. Typically, such a material will be produced by reacting a portion of the side-chains along the polymer backbone with at least one hydrophobic group. Such hydrophobic groups include, for example, alkyl, alkenyl, cycloalkyl, aryl and arylalkyl groups. The alkyl and alkenyl groups may be straight-chained or branched. The hydrophobic groups may contain up to about 22 carbon atoms. In some cases, such groups may be short chain alkyl groups, for example C_{1-6} or C_{1-3} alkyl groups. Methyl, ethyl and propyl groups are particularly suitable.

Natural cellulose materials are typically not water-soluble. Although they contain many hydroxyl groups, these form strong intermolecular hydrogen bonds which

prevent the access of water molecules. Chemical modification of the cellulose to replace some of the hydrogen atoms of the hydroxyl groups by substituents such as methyl groups ($-CH_3$), hydroxypropyl groups ($-CH_2CHOHCH_3$), or hydroxyethyl groups ($-CH_2CH_2OH$) interrupts the intermolecular hydrogen bonding to render the cellulose water-soluble. Examples of modified cellulose materials which are suitable for use in the invention include methyl cellulose (MC), hydroxypropyl methyl cellulose (HPMC) and carboxymethyl cellulose (CMC).

Modified starches which may be used as surfactants in the invention include acetylated starch, hydroxypropyl starch, hydroxy propyl distarch phosphate, starch sodium octenyl succinate, and acetylated oxidised starch. Specific examples of suitable starches include the following food grade starches: E1401 Modified starch; E1402 Alkaline modified starch; E1403 Bleached starch; E1404 Oxidised starch; E1410 Monostarch phosphate; E1412 Distarch phosphate; E1413 Phosphated distarch phosphate; E1414 Acetylated distarch phosphate; E1420 Acetylated starch, mono starch acetate; E1422 Acetylated distarch adipate; E1430 Distarch glycerine; E1440 Hydroxy propyl starch; E1441 Hydroxy propyl starch; E1442 Hydroxy propyl distarch phosphate; E1450 Starch sodium octenyl succinate; and E1451 Acetylated oxidised starch. Preferred for use in the invention are starches having the following E-numbers: E1414, E1420, E1422, E1440, E1441, E1442, E1450 and E1451.

Alginates that are suitable for use as surfactants in the invention are those which have been hydrophobically modified. A chemically modified alginate which may be used in the invention is Propylene Glycol Alginate (PGA). PGA is an ester of alginic acid in which some of the carboxyl groups are esterified with propylene glycol, some are neutralized with an appropriate alkali and some remain free. PGA is available under the E-number E405.

In certain embodiments, the plant-based surfactant for use in the invention may comprise a combination of a protein and a polysaccharide. For example, it may be a polysaccharide-protein complex or conjugate. Alternatively, it may comprise a mixture of a protein and a polysaccharide.

Plant gum exudates are also suitable for use as surfactants in the invention and include, for example, Gum Arabic and Gum Ghatti. Gum Arabic is a substance obtained from two sub-Saharan species of the Acacia tree, Acacia senegal and Acacia seyal. It is widely used in the food industry under the E-number E-414. It is a complex mixture of glycoproteins and polysaccharides predominantly consisting of arabinose and galactose. Gum Ghatti is the dried exudate of the Anogeissus latifolia tree and is a complex, water soluble polysaccharide.

Any of the plant-based surfactants herein described may be used in combination. In one embodiment of the invention, for example, a plant-based protein or derivative thereof as herein described may be used in combination with Gum Arabic. A preferred combination is a pea or bean protein, protein isolate or protein concentrate and a plant gum exudate (e.g. Gum Arabic), for example a combination of faba bean protein and Gum Arabic.

The surfactant (or combination of surfactants) is present in an amount effective to provide the desired stability to the emulsion. The amount will vary dependent on factors such as the precise nature of the surfactant(s), the relative proportions of the oil and aqueous phase, and the presence (and amount) of any other components of the emulsion that may act as an emulsifying agent. Taking account of these factors, an appropriate amount of the plant-based surfactant(s) may readily be determined by those skilled in the art. A suitable amount may, for example, be in the range from 0.1 to 5.0 wt.%, preferably from 0.25 to 4.0 wt.%, particularly from 0.5 to 3.0 wt.%, e.g. from 1.0 to 2.5 wt.% (based on the total weight of the overall composition). For example, the amount of the surfactant(s) may be 1.0, 1.25, 1.5, 1.75, 2.0, 2.25 or 2.5 wt.%, based on the total weight of the composition. When a combination of surfactants is used, their relative amounts may readily be selected by those skilled in the art.

When employing a surfactant that comprises a combination of a plant-based protein or derivative thereof, such as a pea or bean protein, protein isolate or protein concentrate (e.g. faba bean protein), and a plant gum exudate (e.g. Gum Arabic), each component may be present in an amount in the range from 0.5 to 2.0 wt.%, preferably 1.0 to 1.5 wt.% (based on the total weight of the composition). For

example, a surfactant comprising 1.0 to 2.0 wt.% faba bean protein and 0.5 to 1.5 wt.% Gum Arabic may be particularly suitable.

In one set of embodiments, glycerol may be present in the aqueous phase of the emulsion. Advantageously, glycerol may be present in an amount effective to reduce the water activity of the composition and thus reduce microbial growth. Water activity may, for example, be reduced to below about 0.8, for example in the range 0.5 to 0.8, or 0.6 to 0.75, or 0.65 to 0.75. A proportion of the water in the aqueous phase of the emulsion may, for example, be replaced by glycerol. For example, up to 90 wt.% of the water may be replaced by glycerol. In other embodiments, from 10 to 90 wt.%, preferably from 50 to 85 wt.%, e.g. from 55 to 75 wt.% of the water may be replaced by glycerol. When glycerol is present, this can reduce the amount of any preservative agent that may be required to provide a product having an adequate shelf-life. In some cases, it may avoid the need for any preservative agent to be present. As herein described, the presence of sugar alcohols in the aqueous phase also contributes to a reduction in water activity. The amount of glycerol may be adjusted taking into account the amount of any sugar alcohols that may be present. In some embodiments, glycerol may replace the sugar alcohols, or the presence of glycerol may reduce the amount of sugar alcohols.

If present, glycerol may be provided in an amount of up to 60 wt.%, preferably from 20 to 60 wt.%, for example from 30 to 60 wt.% based on the total weight of the composition.

The oil phase of the emulsion will comprise a physiologically tolerable lipid, or a mixture of different physiologically tolerable lipids. Depending on the nature of the lipid (or lipids), the oil phase itself may have nutraceutical and/or pharmaceutical properties. In some embodiments, therefore, the lipids which constitute the oil phase of the emulsion may be the nutraceutical or pharmaceutical agent. Examples of such lipids include, for example, essential fatty acids such as those which are herein described. Alternatively, the oil phase may act as a carrier for a lipophilic pharmaceutical or nutraceutical agent. In this case, the active agent may be dissolved or dispersed in the oil phase.

A range of different lipids are known for oral use in pharmaceutical and/or nutraceutical products and any of these may be used in the oil phase of the emulsions herein described. Sources of lipids include plant oils, such as but not limited to, rapeseed oil, sunflower oil, corn oil, olive oil, sesame oil, palm kernel oil, coconut oil, nut oils (e.g. almond oil or peanut oil), algal oil and hemp oil. Fish oils and lipids obtained from fish oils are also suitable for use in certain compositions according to the invention. Compositions containing these products are acceptable to pescetarians, for example.

Lipids derived from natural products typically comprise a mixture of different lipid components. In one embodiment, the oil phase will thus comprise a mixture of different lipids. For example, it may comprise a mixture of lipids having different chain lengths and/or different degrees of saturation.

Lipids for use in the invention may be liquid, solid or semi-solid at ambient temperature (i.e. at temperatures of about 18°C to about 25°C). Those which are liquid at such temperatures are generally preferred. Any combination of liquid, solid and semi-solid lipids may also be used. Solid lipids having a melting point below about 100°C, preferably below about 70°C, e.g. below about 50°C may be used in the invention. Solid lipids which may be used include butter, solid coconut fraction, cocoa butter or cocoa fat, etc. If desired, the overall melting point of the lipids which make up the oil phase may be modified by mixing different lipids, for example by mixing a solid lipid (e.g. butter) with a liquid oil. An overall melting point in the range from 45 to 50°C may be desirable.

Lipids for use in the invention include, in particular, fatty acids and their derivatives. These include both naturally occurring fatty acids and their derivatives, as well as synthetic analogues. In one embodiment, the oil phase may comprise a mixture of different fatty acids, or fatty acid derivatives.

The hydrocarbon chain of the fatty acid or fatty acid derivative may be saturated or unsaturated, and it may be un-branched or branched. Preferably, it will be un-branched. Typically the hydrocarbon chain will comprise from 4 to 28 carbon atoms, and generally it will have an even number of carbons. Fatty acids differ in their chain length and may be categorized as “short”, “medium”, “long”, or “very

long" chain fatty acids. Those having a hydrocarbon chain of 5 or fewer carbons are referred to as "short-chain fatty acids"; those with a hydrocarbon chain of 6 to 12 carbon atoms are referred to as "medium-chain fatty acids"; those with a hydrocarbon chain of 13 to 21 carbons are referred to as "long-chain fatty acids"; and those with a hydrocarbon chain of 22 carbons or more are referred to as "very long-chain fatty acids". Any of these may be used in the invention.

In one embodiment, the oil phase will comprise a saturated fatty acid, or a derivative of a saturated fatty acid including, but not limited to, any of the derivatives herein described. Medium-chain saturated fatty acids and their derivatives find particular use in the invention. Those having from 8 to 12, e.g. 8, 10 or 12, carbon atoms in the hydrocarbon chain are particularly preferred – i.e. caprylic acid (C8), capric acid (C10) or lauric acid (C12), and any derivatives thereof. Typically, a saturated fatty acid or derivative thereof may be used as a carrier for one or more active components in the oil phase, for example as a carrier for a pharmaceutical or nutraceutical agent.

Saturated fatty acids and their derivatives for use in the invention may be naturally occurring or they may be synthetically produced. Most typically, they will be naturally occurring and thus may be used in the form of mixtures of different fatty acids and/or different fatty acid derivatives. Sources of saturated fatty acids and their derivatives include, for example, coconut oil and palm kernel oil.

In another embodiment, the oil phase may comprise an unsaturated fatty acid or derivative thereof in which the carbon chain contains one or more carbon-carbon double bonds. The double bonds may be in the cis- or trans-configuration, or any combination thereof where more than one double bond is present. Those in which the double bonds are present in the trans-configuration are generally less preferred due to the need to reduce the consumption of so-called "trans-fats" as part of a healthy diet. Fatty acids and their derivatives having cis-configuration double bonds are thus preferred. Mono- and poly-unsaturated fatty acids and their derivatives are well known in the art. Such fatty acids typically will contain 12 to 26 carbons, more typically 16 to 22 carbons, and will have a mono- or poly-unsaturated hydrocarbon chain. They include, in particular, the polyunsaturated fatty acids (PUFAs) such as the essential fatty acids.

Oils which contain long chain, unsaturated fatty acids and their derivatives find particular use in the invention, for example in any composition which is intended for use as a nutraceutical. Particularly important essential fatty acids which may be used include the ω-3, ω-6 and ω-9 fatty acids. Examples of ω-3 fatty acids include alpha-linolenic acid (ALA), stearidonic acid (SDA), eicosatrienoic acid (ETE), eicosatetraenoic acid (ETA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA), tetracosapentaenoic acid and tetracosahexaenoic acid. Examples of ω-6 fatty acids include linoleic acid, gamma-linolenic acid, eicosadienoic acid, dihomo-gamma-linolenic acid (DGLA), arachidonic acid (AA), docosadienoic acid, adrenic acid, docosapentaenoic acid, and calendic acid. Examples of ω-9 fatty acids include oleic acid, eicosenoic acid, mead acid, erucic acid and nervonic acid.

Sources of unsaturated fatty acids and their derivatives include oils obtained from various fish, plant, algae, and microorganism sources. Particularly suitable sources are algae oils and plant oils, however fish oils may also be suitable for those that follow a pescetarian diet. These oils are all rich in ω-3, ω-6 and ω-9 fatty acids. Fish oils may, for example, be obtained from anchovies, sardines and mackerel.

Any known derivatives of the fatty acids may be used in the invention. These include, in particular, the carboxylic esters, carboxylic anhydrides, glycerides (i.e. mono-, di-, or triglycerides) and phospholipids. As used herein the term "derivatives" in the context of a fatty acid also encompasses any pharmaceutically acceptable salt of a fatty acid. Suitable salts are well known to those skilled in the art and include, but are not limited to, the lithium, sodium, potassium, ammonium, meglumine, and diethylamine salts.

Examples of carboxylic acid esters of fatty acids include compounds having a terminal $-CO_2R$ group in which R is a straight-chained or branched alkyl group, typically a short chain alkyl, preferably a C_{1-6} alkyl group, e.g. selected from methyl, ethyl, n-propyl, iso-propyl, n-butyl, sec-butyl and n-hexyl.

Where the fatty acid derivative is a carboxylic anhydride, it may include a terminal $-CO_2COR$ group in which R is a straight-chained or branched alkyl group,

typically a short chain alkyl, preferably a C_{1-6} alkyl group, e.g. selected from methyl, ethyl, n-propyl, iso-propyl, n-butyl, sec-butyl and n-hexyl.

Glycerides are esters derived from glycerol and up to three fatty acids. The fatty acids present may be any of those herein described and thus they may be saturated or unsaturated, for example. In the case of di- and tri-glycerides the fatty acid components may be the same or different. For example, these may be of different chain lengths.

In one embodiment, the lipid carrier for use in the invention may comprise a medium chain triglyceride (MCT). MCTs are triglycerides with two or three medium-chain fatty acids which may be identical or different. Sources of MCTs include coconut oil and palm kernel oil, for example. The fatty acids present in MCTs are typically saturated medium chain fatty acids. MCTs from coconut oil, for example, comprise C_{6-12} fatty acids, predominantly C_8 and C_{10} fatty acids. A typical fatty acid composition of an MCT oil obtained from coconut oil may, for example, comprise: 0.1 wt.% caproic acid (C 6:0), 55 wt.% caprylic acid (C 8:0), 44.8 wt.% capric acid (C 10:0), and 0.1 wt.% lauric acid (C 12:0).

Phospholipids generally consist of a glycerol molecule linked to two fatty acids (the "tail" groups) and to a hydrophilic "head" group which consists of a phosphate group. The phosphate group may be modified by linkage to choline, ethanolamine or serine. In one embodiment, the oil phase may be constituted in whole or part by a phospholipid, for example a plant lecithin.

The amount of oil present in the compositions of the invention will be dependent on factors such as the nature of the oil, the nature and desired loading level of any pharmaceutical or nutraceutical that may be present, etc. and can be varied according to need. The oil phase may, for example, constitute from 5 to 50 wt.%, preferably from 10 to 45 wt.%, for example from 15 to 40 wt.%, from 15 to 30 wt.% or from 20 to 25 wt.% of the gelled oil-in-water emulsion.

As will be understood, in the compositions of the invention the oil provides the discontinuous phase within a continuous aqueous phase which is gelled. The oil is thus dispersed throughout the gelled aqueous phase in the form of oil droplets (also

referred to herein as oil "particles"). Gelling of the aqueous phase provides a stable emulsion which prevents coalescence of the droplets of oil, for example due to the prevention of physical collisions between droplets. When preparing the gelled emulsions, the plant-based surfactant will initially be dissolved in the aqueous phase but migrates to the oil-water interface where it serves to stabilise the oil phase. Although not wishing to be bound by theory, it is believed that an uneven distribution of the large molecules of the surfactant around the oil droplets serves to provide a friction layer which provides a "semi active filler" effect.

The size of the oil particles in the gelled oil-in-water emulsion is not particularly limited. For example, oil particles having a volume-based size in the range from about 100 nm to about 100 µm, preferably from about 500 nm to about 75 µm, in particular from about 750 nm to about 50µm, e.g. from about 1000 nm to about 40 µm may be provided. Volume-based average particle sizes may range from about 5 to 50 µm, preferably from about 5 to 30 µm, e.g. about 5 to 25 µm. "Volume-based average" as used herein refers to the volume moment mean or De Brouckere Mean Diameter (also known as the "D[4,3]" value). This reflects the size of those particles which constitute the bulk of the sample volume and is most sensitive to the presence of large particles in the size distribution.

An essentially homogenous size distribution of oil particles may be desirable. The D_{90} value indicates the size value which 90% of the oil particles meet out of the entirety of all of the oil droplets. D_{90} values may range from 15 to 80 µm, preferably 25 to 65 µm, in particular from 30 to 50 µm. Correspondingly, the D_{50} and D_{10} value, respectively, indicate the size value which 50% and 10% of the oil droplets meet out of the entirety of all of the oil droplets. D_{50} values may range from 10 to 45 µm, in particular from 15 to 35 m, e.g. from 18 to 25 µm. D_{10} values may range from 0.5 to 20 µm, in particular from 3 to 15 µm, e.g. from 5 to 10 µm.

Lipid droplet size and size distributions can be determined using methods and apparatus conventional in the art, for example using a Malvern Mastersizer 3000 (Worcestershire, UK) connected to a Hydro MV, wet dispersion unit (Malvern,Worcestershire, UK). Analysis of the data may be performed using the manufacturer's software (Mastersizer 3000, v1.0.1). Testing may be carried out by dissolving and diluting the gelled emulsion in a suitable solvent (1:100) at 50°C.

Suitable solvents include Milli-Q water and a 10% (v/v) HCl solution (the latter may minimize flocculation during testing). The refractive index of water and corn oil is set to 1.33 (solvent) and 1.47 (dispersed phase), respectively, and the absorption index of the dispersed droplets set to 0.01. To avoid multiple scattering or low intensity of the scattered light, the dissolved emulsion is added to the dispersion unit (containing ~125 mL water), until an obscuration of approximately 10% is obtained.

The size and size distribution of the oil particles may be varied. If desired, size reduction of the oil particles can be achieved by various different means, for example by mechanical processes or by chemical processes involving the selection of smaller lipid molecules, or indeed by a combination of these approaches. Chemical methods suitable for achieving a size reduction of the oil particles may involve the selection of a particular type of lipid (or combination of lipids) capable of forming smaller oil droplets. Certain oils, such as MCTs for example have a tendency to produce a finer dispersion of oil droplets. Mechanical reduction involves the use of shear forces to break down larger oil droplets into smaller nano-scale particles. Smaller particles may thus be produced by suitable adjustments to the method used to produce the emulsion, for example by varying the shear force and/or the duration of mixing of the oil and aqueous phases. The use of higher shear forces and/or longer mixing times will produce smaller particles of oil. Suitable shear may be achieved, for example, using a conventional homogenizer such as a rotor-stator mixer, e.g. an Ultra-turrax® homogenizer. A problem often encountered in mechanical processes for the production of oil-in-water emulsions is the re-aggregation (i.e. coalescence) of the particles, but this is addressed in the invention by the use of a gelled aqueous phase which serves to stabilize the emulsion and the use of a surfactant which reduces the energy required for emulsification (by reducing interfacial tension) and which protects the droplets against re-aggregation.

The aqueous phase (i.e. continuous phase) of the gelled oil-in-water emulsion may constitute from 50 to 95 wt.%, preferably from 55 to 90 wt.%, for example from 60 to 85 wt.%, from 70 to 85 wt.%, or from 75 to 80 wt.% of the composition.

In addition to water, the gelling agent(s) and the surfactant(s), other physiologically tolerable materials may also be present in the aqueous phase, for example, pH modifiers (e.g. buffering agents), viscosity modifiers (e.g. thickening agents, plasticizers), sweeteners, bulking agents (i.e. fillers), anti-oxidants, aromas, flavouring agents, and colouring agents. The nature and concentration of any such materials may readily be determined by those skilled in the art.

The presence of bulking agents (i.e. fillers) in the aqueous phase aids in reducing water activity and thus in reducing microbial growth. Water activity may, for example, be reduced to below about 0.8, for example in the range 0.5 to 0.8, or 0.6 to 0.75, or 0.65 to 0.75. The amount and type of bulking agents may readily be selected by those skilled in the art. Suitable examples include, but are not limited to, sugar alcohols, sugars and mixtures thereof. Suitable sugar alcohols include sorbitol and xylitol and mixtures thereof. Sugars which may be used include trehalose, sucrose, glycerol and mixtures thereof. Bulking agents may constitute from 45 to 70 wt.%, preferably 50 to 65 wt.%, e.g. 55 to 60 wt.%, based on the aqueous phase. In some cases, the selected bulking agent(s) may also act as sweetening agents depending on their concentration. For example, the compositions according to the invention may contain xylitol, e.g. as 0.5 to 50 wt.%, preferably 1 to 40 wt.%, e.g. 15 to 40 wt.%, in order to improve taste.

Where a sweetener is included in the aqueous phase, this will typically be selected from natural sweeteners such as sucrose, fructose, glucose, reduced glucose, maltose, xylitol, maltitol, sorbitol, mannitol, lactitol, isomalt, erythritol, polyglycitol, polyglucitol, glycerol and stevia, and artificial sweeteners such as aspartame, acesulfame-κ, neotame, saccharine, and sucralose. The use of non-cariogenic sweeteners is preferred.

In one embodiment, viscosity modifiers may also be provided in the aqueous phase. Suitable viscosity modifiers include other hydrocolloids such as starch, modified starch (e.g. hydroxy ethyl starch, hydroxy propyl starch), xanthan, galactomannans (e.g. guar gum and locust bean gum), gum karaya, gum tragacanth, and any combination thereof. As will be understood, a viscosity modifier may possess some surface-active properties and may additionally aid in stabilisation of the emulsion. The thickening effect of the viscosity modifier

depends on the type of material (e.g. hydrocolloid) used and its concentration, the other components and the pH of the formulation, etc. but suitable amounts may readily be determined by those skilled in the art. Typical amounts of any viscosity modifier which may be present may range from 0.1 to 5 wt.% of the overall composition, preferably from 0.2 to 2.5 wt.%, for example from 0.5 to 2.0 wt.%.

Flavoring agents may be present in the compositions and may, for example, aid in taste masking certain lipids such as those which contain omega-3 fatty acids. Suitable flavors include, but are not limited to, citrus flavors, for example orange, lemon or lime oil.

pH modifiers may readily be selected by those skilled in the art and include food grade acids such as citric acid. Buffering agents may also be used to adjust pH and include organic acid / base buffering systems. Suitable buffering agents are well known in the art and include, for example, sodium citrate and malic acid, etc. The pH of the aqueous phase of the emulsion may be adjusted to be in the range from 2 to 8, particularly 3 to 7, preferably 3.5 to 6, for example 4 to 5.

Where antioxidants are present in the aqueous phase these will be water soluble and include, for example, ascorbic acid, citric acid and salts thereof such as sodium ascorbate. Depending on the choice of oil, these may be supplied in a form which contains an antioxidant such as vitamin E, for example. If present, the amount of any anti-oxidant(s) may be up to 3 wt.% of the overall formulation, e.g. up to 1 wt.%.

In addition to the lipid(s), the oil phase of the emulsion may also if desired contain physiologically tolerable lipid soluble materials, for example pharmaceutically acceptable agents, anti-oxidants (e.g. vitamin E), flavorings, and coloring agents.

In some embodiments, additional physiologically active agents may also be present in the gelled emulsions herein described. These may be provided in the aqueous and/or oil phases and may be dissolved and/or dispersed in one or both of these phases. Other actives which may be present in the oil phase include fat soluble active agents.

In one embodiment, the gelled oil-in-water emulsions according to the invention may comprise, consist essentially of, or consist of, the following components:

(a) water;
(b) at least one plant-based surfactant;
(c) one or more physiologically tolerable lipids;
(d) agar;
(e) one or more bulking agents;
(f) optionally one or more pH modifiers;
(g) optionally one or more viscosity modifying agents (e.g. thickening agents or plasticisers); and
(h) optionally one or more additional physiologically active agents.

By "consisting essentially of" it is intended that the emulsions will be substantially free from (e.g. free from) other components which materially affect their properties. By "consists of" it is intended that the emulsions will be substantially free (e.g. free from) from any other components than those listed.

In one set of embodiments the compositions of the invention may be provided in the form of a dose unit. By "dose unit" it is intended that the composition will be taken orally by the subject (e.g. administered to a patient) "as received", i.e. it will not be broken or cut before oral delivery. The weight of the dose unit will therefore be such that the composition is suitable for delivery in this way. For example, it may have an overall weight in the range from 50 to 3,000 mg, e.g. 250 to 3,000 mg or 500 to 2,500 mg, especially 100 to 2,000 mg, e.g. 750 to 2,000 mg, particularly 100 to 1,500 mg, more particularly 400 to 1,500 mg, more especially 400 to 1,000 mg. In one set of embodiments, the dose units will generally be quite large, e.g. having a mass of from 400 to 3,000 mg, e.g. 600 to 1,500 mg. The overall dose unit weight may be selected as required. For example, it may be scaled up or down dependent on the nature of the selected active components and their intended dose.

Each dose unit will typically consist of a self-supporting, gelled oil-in-water emulsion as herein described. As will be understood, in this case the dose unit will contain only the defined oil and aqueous phases, i.e. it will be free from any other components. Individual dose units may be prepared from a larger piece of the

gelled emulsion which is divided, e.g. by cutting. More typically, however, each dose unit will be formed by extrusion or moulding from a liquid emulsion, or incompletely gelled emulsion, prior to gelation (i.e. above the gelling temperature of the gelling agent).

Alternatively, a core of the gelled oil-in-water emulsion may be provided with a suitable coating of a physiologically tolerable coating material. Such coatings may be of the type conventional in the pharmaceutical and nutraceutical industry and may be applied by any conventional means, for example by dipping or spraying. In one set of embodiments, the gelled oil-in-water emulsions herein described may therefore be provided with a coating. For example, these may be provided within a capsule shell which dissolves in the mouth. Viewed from another aspect the invention thus provides an orally administrable capsule comprising a capsule shell enclosing a gelled oil-in-water emulsion as herein described.

In the capsules of the invention, the shell may be of any physiologically tolerable material but will typically be a sugar, a biopolymer or a synthetic or semi-synthetic polymer which is soluble or disintegrable in saliva or fluid within the gastrointestinal tract. The shell may be soft, but is preferably substantially rigid. Particularly desirably, the capsules will have the consistency of a "jelly bean". The shell will preferably be of a material and a thickness to prevent oxidation of the contents. The shell may comprise a sugar or cellulose, for example sorbitol. The use of sugars and cellulose as capsule shell materials is well-known in the pharmaceutical and nutraceutical fields.

The capsule shell material may thus typically be a sugar, e.g. sucrose, fructose, maltose, xylitol, maltitol or sorbitol, but may additionally contain hydrocolloid materials such as for example carageenan, alginate, pectin, cellulose, modified cellulose, starch, modified starch, gum arabic, etc. The capsule shell may contain other ingredients such as, for example, artificial sweeteners, colors, fillers, flavors, antioxidants, etc.

The capsule shell may be pre-formed such that the oil-in-water emulsion can be filled into the shell either as a liquid, or once set. Alternatively a shell precursor

(e.g. a solution) may be coated onto the set emulsion, for example using standard coating techniques. If desired the capsule may be further coated, e.g. with a wax.

Preparation of the gelled oil-in-water emulsions herein described may be carried out by emulsification of the aqueous and oil phase components. It will be understood that emulsification is carried out under conditions in which the aqueous phase is a liquid (for example a viscous liquid), i.e. prior to the formation of a gel. Emulsification will thus be carried out at a temperature above the sol-gel transition temperature of the agar gelling agent. Subsequent cooling of the emulsion below its sol-gel temperature results in the desired gelled emulsion.

Prior to emulsification, any selected active agents may be added to the oil and/or aqueous phase of the composition. This may be done, for example, by dissolving the active in the selected oil or in the aqueous phase prior to forming the emulsion. Alternatively, the selected active agent(s) may be added to a mixture of the aqueous and oil phase components prior to emulsification. During the emulsification process, the active agents will typically migrate to the oil or aqueous phase depending on their hydrophilic / lipophilic characteristics.

Emulsion formation may be effected by conventional techniques and using known equipment, for example a homogenizer based on the rotor-stator principle. The speed and duration of stirring may be adjusted as required, for example it may be varied to achieve the desired shearing force to provide the desired droplet size.

Emulsification will generally be carried out under a controlled atmosphere in order to avoid oxidative degradation of the lipid and/or any active agents. For example, emulsification may be carried out in the presence of a non-oxidising gas such as nitrogen. De-gassing to remove air bubbles may also be carried during the production process, for example prior to mixing the components of the emulsion, once the liquid emulsion has been formed, prior to packaging of the set emulsion, etc. De-gassing may be carried out using any conventional means such as the application of a vacuum, or sparging with a non-oxidising gas (e.g. nitrogen). After emulsification and gelling, the emulsion may be dried to reduce the water content. If dried, however, it will still retain a continuous gelled aqueous phase as herein described and a water content within the limits herein defined.

The gelled oil-in-water emulsions will typically be provided in dose unit form as herein described. Individual dose units may be formed by methods such as molding, extrusion or cutting. Typically, however, the dose units may be formed by filling of the liquid emulsion into molds, e.g. the individual molds of a blister pack which is then sealed. The dose units will typically be in tablet or lozenge form.

Methods for preparation of the gelled oil-in-water emulsions herein described form a further aspect of the invention. Viewed from a further aspect, the invention thus provides a method for preparing an orally administrable, gelled oil-in-water emulsion, said method comprising: forming an oil phase which comprises one or more physiologically tolerable lipids; forming an aqueous phase comprising a gelling agent which is agar; combining said oil phase and said aqueous phase to form an oil-in-water emulsion in the presence of a plant-based surfactant as herein described; and allowing said emulsion to gel. Optionally, prior to or after allowing the emulsion to gel, the emulsion may be divided into individual dose units.

The dose units are preferably individually packaged in air-tight containers, e.g. a sealed wrapper or more preferably a blister of a blister pack. In another aspect, the invention thus provides a package comprising an air-tight and light-tight compartment containing one dose unit of a composition according to the invention. By excluding both air (i.e. oxygen) and light from the packaged dose unit, long term stability of the active components is enhanced.

The packages according to the invention are preferably provided in the form of blister packs containing at least two dose units, e.g. 2 to 100, preferably 6 to 30 dose units. The blister pack will generally comprise a metal, metal/plastic laminate or plastic sheet base having molded indentations in which the dosage form is placed. The pack is normally sealed with a foil, generally a metal or a metal/plastic laminate foil, for example by applying heat and/or pressure to the areas between the indentations. The use of a metal or metal/plastic laminate to form the blister pack serves to prevent air (i.e. oxygen), light and humidity from penetrating the contents of the blister pack thus enhancing the stability of active component(s). The packages according to the invention are preferably filled under a non-oxidising

gas atmosphere (e.g. nitrogen) or are flushed with such a gas before sealing.

The use of agar as a gelling agent in the compositions according to the invention provides additional advantages in relation to packaging of individual dose units in a blister pack and their removal by the end user. When an emulsion in liquid form is used to fill the indentation of the blister pack (i.e. prior to gelling), it will be in intimate contact with the inner surface of the indentation. After setting of the dose unit and sealing of the blister pack, it is important that the dose unit can easily be removed from the blister pack. The presence of gelatin, first developed as a glue, in known gelatin-based compositions can give rise to the difficulty in removing these from certain surfaces, such as those made from plastic materials, especially when a liquid emulsion containing the gelatin has been allowed to set in contact with the surface. In such cases, once set, the dose unit tends to adhere to the surface and must be torn away often causing the dose unit to fragment in the process which is not acceptable. When packaging any conventional gelatin-based dose unit, it is necessary for the internal surface of a blister pack to be coated with a suitable release agent such as a neutral oil or fat. Specially developed blister pack materials having release agents incorporated onto their internal surfaces are available but add to the cost of the packaging process. The use of a release agent also leads to a surface coating of the agent on the dose unit once it has been removed from the blister pack and this can give rise to an unpleasant feel or taste of the product.

In contrast to the use of gelatin, agar-based dose units do not adhere to conventional blister pack materials. This means that standard materials can be used, including metal/plastic laminate or a plastic film over which a plastic/metal foil laminate is heat sealed. Suitable blister trays with pre-formed cavities may, for example, be formed from laminated materials such as Tekniflex® Aclar® VA10600 (TekniPlex), Perlalux® (Perlen Packaging), Formpack® (Amcor), and Regula® (Constantia Flexibles). Such materials do not have any surface coating containing a release agent.

In one embodiment, the dose units of the invention may thus be packaged in a blister pack having an internal surface which is not coated with any release agent.

Such blister packs containing a dose unit as herein described form a further aspect of the invention.

The gelled oil-in-water emulsions according to the invention find use both as pharmaceuticals, i.e. for therapeutic purposes, and as nutraceuticals to maintain or augment health and/or general well-being of a human or animal subject. For this purpose, it is intended that they are taken orally, lightly chewed in the mouth and then swallowed. It is not intended that they should remain in the mouth or need to be chewed for an extended period. Due to their soft texture, light chewing is sufficient to fragment the dosage form into smaller pieces which are easily swallowed. What the Applicant has surprisingly found is that the gelled oil-in-water emulsions according to the invention have a much better mouthfeel than pure aqueous agar gels. Whereas aqueous agar gels are brittle and 'fracture' in the mouth on chewing, the emulsions herein described have a greater resistance to deformation when chewed and are less susceptible to fracturing. This provides a much more acceptable chewing experience for the patient or consumer.

When used as nutraceuticals, for example, the compositions herein described may be used as a supplement (e.g. as a dietary supplement) for maintaining the general health and/or well-being of a subject. Any agent known for its nutraceutical effects may be provided in the compositions and suitable agents are well known in the art. Suitable nutraceuticals include, but are not limited to, any of the following: essential fatty acids (e.g. mono and poly-unsaturated fatty acids), essential amino acids (e.g. taurine, tryptophan, tyrosine, cysteine and homocysteine), vitamins (e.g. vitamins A, B1-B12, C, D, E, K and folate), minerals (e.g. iodine, selenium, iron, zinc, calcium and magnesium), flavonoids, carotenoids (e.g. beta carotene, alpha carotene, luteine, zeoxantaine, xanthophylls and lycopene), phytosterols, sapponins, probiotics, dietary fibres (e.g. insoluble fibre and beta-glucans), and plant extracts (e.g. aloe vera, evening primrose oil, garlic, ginger, ginseng, green tea, caffeine and cannabinoids). Where magnesium or calcium are present, these will generally be used in the form of their phosphate salts.

In particular, the gelled oil-in-water emulsions herein described may be used as a source of one or more essential fatty acids, such as PUFAs or their esters, e.g. omega-3, omega-6 and/or omega-9 fatty acids and their ester derivatives.

Examples of omega-3 acids include α-linolenic acid (ALA), stearidonic acid (SDA), eicosatrienoic acid (ETE), eicosatetraenoic acid (ETA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA), tetracosapentaenoic acid and tetracosahexaenoic acid. Examples of omega-6 acids include linoleic acid, gamma-linolenic acid, eicosadienoic acid, dihomo-gamma-linolenic acid (DGLA), arachidonic acid (AA), docosadienoic acid, adrenic acid, docosapentaenoic acid, and calendic acid. Examples of omega-9 acids include oleic acid, eicosenoic acid, mead acid, erucic acid and nervonic acid. Omega-3 acids are especially preferred, particularly EPA and DHA.

The health benefits of essential fatty acids, in particular omega-3 fatty acids, are well known. For example, these may lower triglyceride levels and/or lower cholesterol levels. Omega-3 fatty acids are vital to everyday life and health. The beneficial effects of EPA and DHA on lowering serum triglycerides are well known. They are also known for other health benefits such as cardio-protective effects, e.g. in preventing cardiac arrhythmias, stabilising atherosclerotic plaques, reducing platelet aggregation, and reducing blood pressure. They find use therefore in treating and/or preventing vascular disease. Other benefits of omega-3 fatty acids include the prevention and/or treatment of inflammation and neurodegenerative diseases, and improved cognitive development and function.

The essential fatty acids may form part or the whole of the oil phase in the gelled emulsion, preferably at least 10% wt, more especially at least 50% wt, particularly at least 80% wt. of that phase. They may be used as single compounds or as compound mixtures, e.g. plant or marine oils. The free fatty acids, the monoacyl glycerides and diacylglycerides may be prepared by full or partial hydrolysis of triacylglycerides, for example acid, base, or enzyme-catalysed hydrolysis, e.g. using lipases such as pancreatic lipases and/or lipases which may be produced from bacteria as fermentation products. Alkyl esters of essential fatty acids may be prepared by transesterification using the appropriate alkanol or by esterification of the free fatty acid with that alkanol. Where a free fatty acid is used, this may be in acid form or salt form (e.g. wholly or partially in salt form), and preferably constitutes 5 to 75% wt, especially 10 to 35% wt. of the essential fatty acid in the oil phase. Salt forms may be preferred.

The gelled oil-in-water emulsions herein described also find use as pharmaceuticals in the treatment or prevention of a range of medical conditions which are responsive to the chosen active agent(s). As will be appreciated, the nature of such conditions will be dependent on the selected active agent(s), but can readily be determined by those skilled in the art.

Any drug substance having a desirable therapeutic and/or prophylactic effect may be used. This includes drug substances which are lipophilic or hydrophilic.

Classes of suitable drug substances include, but are not limited to, any of the following: analgesics; anti-inflammatories; anti-cancer agents; cardiovascular agents; biological agents; anti-allergy agents (e.g. antihistamines); decongestants; anti-nausea agents, drugs affecting gastrointestinal function; drugs acting on the blood and blood-forming organs; drugs affecting renal and cardiovascular function; anti-fungal agents; urological agents; hormones; antimicrobial agents, anti-epileptical agents; psycholeptical agents; antipsychotic agents; psychoanaleptical agents; anticholinesterase agents; and carotenoids.

Examples of specific drug substances which may find use in the compositions of the invention include: temazepam; diphenhydramine; zolpidem; triazolam; nitrazepam; testosterone; estradiol; progesterone; benzodiazepines; barbiturates; cyclosporine; insulin; calcitonin; dextromethorphan; pseudoephedrine; phenylpropanolamine; bromocryptine; apomorphine; selegiline; amitriptyline; dextroamphetamine; phentermine; mazindol; compazine; chlorpromazine; perphenazine; fluoxetine, buspirone; clemastine; chlorpheniramine; dexochlorpheniramine; astemizole; loratadine; paracetamol; ketoprofen; naproxen; ibuprofen; sodium acetazolamide, acetyl salicylic acid, aminophylline, amiodarone hydrochloride, ascorbic acid, atenolol, bendroflumethiazide, calcium folinate, captopril, cetrizine hydrochloride, chloramphenicol sodium succinate, chlorpheniramine maleate, chlorpromazine hydrochloride, cimetidine hydrochloride, ciprofloxacin hydrochloride, clindamycin hydrochloride, clonidine hydrochloride, codeine phosphate, cyclizine hydrochloride, cyclophosphamide, sodium dexamethasone phosphate, sodium dicloxacillin, dicyclomide hydrochloride, diltiazem hydrochloride, diphenhydramine hydrochloride, disopyramide phosphate, doxepin hydrochloride, enalapril maleate, erythromycin ethylsuccinate, flecanide

acetate, fluphenazine hydrochloride, folic acid, granisteron hydrochloride, guafenesin, haloperidol lactate, hydralazin hydrochloride, hydrochloroquine sulfate, hydromorphone hydrochloride, hydroxyzine hydrochloride, sodium indomethacin, isoniazid, isoprenaline hydrochloride, ketorolac trometamol, labetalol hydrochloride, lisinopril, lithium sulfate, mesoridazine benzylate, methadone hydrochloride, methylphenidate hydrochloride, methylprednisolone sodium succinate, metorprolol tartrate, metronidazole hydrochloride, metyldopa, mexiletine hydrochloride, molidone hydrochloride, morphine sulfate, naltrexone hydrochloride, neomycin sulfate, ondanstreon hydrochloride, orciprenaline sulfate, sodium oxacillin, oxybutynin chloride, oxycodone hydrochloride, paracetamol, penicillamine, pentoxifylline, petidine hydrochloride, sodium phenobarbital, potassium phenoxymethylpenicillin, phenylephrine hydrochloride, sodium phenytoin, potassium iodide, primaquine phosphate, procainamide hydrochloride, procarbazine hydrochloride, prochlorperazine maleate, promazine hydrochloride, promethazine hydrochloride, propranolol hydrochloride, pseudoephedrine hydrochloride, pyridostigmine bromide, pyridoxine hydrochloride, ranitidine hydrochloride, salbutamol sulfate, sodium ethacrynate, sotalol hydrochloride, sumatripan succinate, terbinafine hydrochloride, terbutaline sulfate, tetracycline hydrochloride, thioridazine hydrochloride, thiothixene hydrochloride, trifluoperazine hydrochloride, triprolidine hydrochloride, sodium valproate, vancomycin hydrochloride, vancomycin hydrochloride, verapamil hydrochloride, sodium warfarin, astaxanthin, lutein, CoQ10 and fenofibrate.

The quantity of drug substance per unit dose of the compositions of the invention will conveniently be in the range of 10 to 100% of the recommended daily dose for an adult or child.

Viewed from another aspect, the invention thus provides a gelled oil-in-water emulsion as herein described for use in therapy.

Viewed from still another aspect, the invention provides a gelled oil-in-water emulsion as herein described which contains at least one pharmaceutically active component for oral use in the treatment of a condition responsive to said pharmaceutically active component.

In another aspect the invention provides the use of a pharmaceutically active component in the manufacture of a medicament for oral use in the treatment of a condition responsive to said pharmaceutically active component, wherein said medicament is provided in the form of a gelled oil-in-water emulsion as herein described.

Corresponding methods of medical treatment form a further aspect of the invention. Viewed from a yet further aspect, the invention thus provides a method of treatment of a human or non-human animal subject (e.g. a patient) to combat a condition responsive to a pharmaceutically active agent, said method comprising the step of orally administering to said subject a pharmaceutically effective amount of said agent in the form of a gelled oil-in-water emulsion as herein described.

In another aspect the invention provides the use of a gelled oil-in-water emulsion as herein described as a nutraceutical. Corresponding methods of administering the gelled oil-in-water emulsion in order to achieve a nutraceutical effect also form part of the invention.

Viewed from another aspect the invention thus provides a method of administering an active agent to a human or non-human animal subject to enhance and/or maintain said subject's health or wellbeing, said method comprising the step of orally administering to said subject a nutraceutically effective amount of an active agent in the form of a gelled oil-in-water emulsion as herein described.

In another aspect the invention provides the use of a gelled oil-in-water emulsion as herein described as a nutraceutical.

When used in any of the above treatments or methods, or as nutraceutical supplements or pharmaceutical formulations, an effective amount of the active agent can readily be determined.

The effective dose level for any particular subject will depend on a variety of factors including the disorder and its severity, the identity and activity of the particular composition, the age, bodyweight, general health of the subject (e.g. patient), timing of administration, duration of treatment, other drugs being used in combination with

the treatment, etc. It is well within the skill of those in the art to select the desired dose to achieve the desired therapeutic effect.

The invention will now be described further with reference to the following non-limiting Examples and the accompanying figures in which:

Figure 1 shows the dynamic storage modulus (G' max) for agar-based gelled oil-in-water emulsions containing different surfactants.
Figure 2 shows the dynamic storage modulus (G' max) for gelled oil-in-water emulsions according to the invention.
Figure 3 shows the hardness (force) of gelled oil-in-water emulsions according to the invention measured in accordance with a texture profile analysis (TPA) test.
Figure 4 shows the dynamic storage modulus (G' max) for gelled oil-in-water emulsions according to the invention.
Figure 5 shows the hardness (force) of gelled oil-in-water emulsions according to the invention when subjected to large scale deformation.
Figure 6 shows the measured water activity of aqueous agar gels with increasing glycerol content.
Figure 7 shows the hardness (force) of gelled oil-in-water emulsions according to the invention compared to a pure agar gel.
Figure 8 shows the hardness (force) of gelled oil-in-water emulsions containing gelatin as the gelling agent.

Examples

Test Methods:

1. Rheological characterisation of agar and agar emulsion gels

1a - Small Scale Deformation

Rheological analyses on the gels were performed with a rheometer (Malvern Kinexus ultra+, Westborough, United States). The lower plate was KNX0127, 50 mm diameter curved sandblasted lower plate. The upper geometry was CP4/40 40 mm diameter 4° angle cone for gelatin emulsion gels and serrated PP40X SW1648 SS for agar gels and agar emulsion gels. Instrument calibration (zero gap) was performed prior to analysis. After gel preparation, approximately 2 grams of gel

was placed on the lower plate, which was heated up to 60°C. The rheometer was operated in 0.1% shear strain controlled mode and the frequency was set to 1 Hz. The chosen strain was confirmed to be within the linear viscoelastic region for all samples. In order to avoid evaporation, the gelatin emulsion gel samples were covered with silicone oil (10 cS fluid, Dow Corning, UK) prior to measurement. The viscoelastic properties of the sample were obtained by using a temperature gradient of 2°C/min, with a start and end temperature at 60°C and a holding time of 15 min at 20°C for the gelatin emulsion gels. For agar emulsion gels, the end temperature was 90°C and oscillation continued for 10 minutes at 90°C. The results were analyzed using rSpace for Kinexus software. The gelling and melting temperatures of the samples were estimated as the temperature at which the phase angle corresponded to 45° in the cooling and heating process, respectively. The maximum storage modulus (G') (Pa) was determined as the highest measurement point during curing at 20°C.

1b - Large Scale Deformation

Texture properties of the gels were analysed with TA.XT plusC Texture Analyser (Stable Micro Systems Ltd., UK). Upon preparation, the gels were cast using cylindrical molds of standard dimensions (19.6 mm height, 8 mm diameter). The gels were cured at ambient temperature for 18 hours prior to analysis. Single compression analysis and the standard texture profile analysis (TPA) were performed using a 5 kg load cell. A P/35 35 mm diameter cylinder aluminum probe supplied by Stable Micro Systems Ltd. was used. For the 75% large and strain single compression, pre-test and post-test speeds were 2 mm/sec, while the test speed was 0.5 mm/sec and the trigger force was 5 grams. Strain height was measured automatically during compression. Max stress (g) and strain at failure (%) data was obtained from the fraction moment of the gels. Gradient (N/m) was calculated by the ratio of force at 2% and 3% strain. Young's modulus (N/m^2) was calculated from gradient by following equation:

$$Young's\ modulus\ (N/m^2) = \frac{Gradient\ (N/m) \times height\ of\ the\ gel\ (m)}{Area\ of\ the\ gel\ (m^2)}$$

Area of the gel is the contact area of the gel with the probe.

2. Texture profile analysis (TPA test)

The standard TPA was carried out at 20% strain double compression at room temperature applying a TA.XT plusC Texture Analyser (Stable Micro Systems Ltd., UK) using a 5 kg load cell and a P/35 aluminum probe. Cylindrical molds of standard dimensions (19.6 mm height, 8 mm diameter) were used. The gels were cured at ambient temperature for 18 hours prior to analysis. Pre-test, test and post-test speeds were 1 mm/sec and the trigger force was 5 grams. Strain height was measured automatically during compression. Hardness, adhesives, resilience, cohesion, springiness, gumminess and chewiness parameters were measured. The data were analyzed with the Exponent connect software.

3. Syneresis measurements

Syneresis measurements were based on weight loss of the gels. The gel was weighed and sealed with an air- and moisture-tight aluminum foil. Upon freezing at -20°C and thawing at ambient temperature, and after removing excess liquid, the gel was weighed again and the difference in gel weight was normalized to percentage loss.

4. Water activity measurements

Water activity was measured with HygroPalm HC2-AW (Rotronic, Switzerland) at ambient temperature. The sample was placed into the measurement chamber and the water activity was recorded after 45 minutes.

Example 1 – Gelled oil-in-water compositions and preparation method

Composition:

Typical gelled oil-in-water compositions according to the invention are listed in the following table. It will be understood that any component which may be present in an amount of 0 wt.% is optional.

Component	Wt.%
Agar (gelling agent)	0.5 – 2.5
Plant-based surfactant(s)	0.25 – 3.5
Bulking agent(s), e.g. sugar alcohol(s)	30 – 60
pH modifier(s)	0 – 6

Oil(s)	10 – 50
Viscosity modifier(s)	0 – 5
Plasticiser(s), e.g. glycerol	0 – 60
Anti-foaming agent(s)	0 – 0.5
Anti-Oxidant(s)	0 – 3
Sweetening agent(s)	0 – 3
Flavouring agent(s)	0.05 – 3
Colouring agent(s)	0.001 – 3
Pharmaceutical agent(s)	0 – 10
Nutraceutical agent(s)	0 – 10
Water	to 100

Method of preparation:

In the following method, the pH modifier is an organic acid / base buffer system consisting of trisodium citrate and malic acid, and the plasticiser (when present) is glycerol.

1. Mix agar, sugar alcohols and any sweetening agent(s) into a homogeneous powder mixture.
2. Weigh sterile water into a bottle and add the powder mixture to the water.
3. Place bottle in a water bath at 90°C and mix with magnetic stirring for 30 minutes at 100 rpm. If glycerol is used, heat glycerol separately for 30 minutes at 60°C.
4. Reduce the temperature to 60°C and mix the water phase with magnetic stirring for an additional 30 minutes (approx. 60 minutes in total). If glycerol is used, add the heated glycerol to the mixture with a syringe.
5. In a beaker, mix the oil together with any flavouring and/or colouring agent(s) and pre-heat to 50°C for 30 minutes (at minute 40 of total 60 minutes).
6. When the ingredients are completely dissolved, slowly add the surfactant and trisodium citrate to the water phase. Where the surfactant contains any plant protein, it is added at a temperature below the denaturation temperature of the plant protein. Mix for 10 minutes and slowly add malic acid (carefully and gradually). Mix the mass for 10 more minutes.
7. If using an anti-foaming agent, add half of this agent and leave for 1 minute without stirring.

8. Weigh the bottle containing the water phase and vacuum the mass. Add the lost water (heated) and mix for 1 minute.
9. Add the oil phase into the agar mass (water phase) and homogenise the two phases for approx. 10 minutes using a high speed blender, such as an Ultra-Turrax.
10. If appropriate, add the second half of the (heated) anti-foaming agent and leave for 1 minute without stirring.
11. Weigh the bottle and vacuum the mass. Add the lost water (heated) and mix for 1 minute.
12. If desired, fill the resulting emulsion into blisters of a blister pack and seal.

Example 2 – Gelled oil-in-water emulsion – typical formulation

Component	**Wt.%**
Agar (gelling agent)	0.5 – 2.5
Plant-based surfactant	0.25 – 3.5
Xylitol (bulking agent)	20 – 40
Sorbitol (bulking agent)	10 – 25
Malic acid (pH modifier)	0 – 2
Trisodium citrate (pH modifier)	0 – 4
Oil	10 – 50
Gum Arabic (surfactant)	0 – 2
Locust Bean Gum (viscosity modifier)	0 – 3
Glycerol (plasticiser)	0 – 60
Anti-foaming agent(s)	0 – 0.5
Sweetening agent(s)	0 – 3
Flavouring agent(s)	0.05 – 3
Colouring agent(s)	0.001 – 3
Anti-oxidant(s)	0 – 3
Water	to 100

The emulsion can be prepared according to the general method in Example 1.

Example 3 – Gelled oil-in-water emulsion containing algae oil

Ingredient	Wt.%
Agar HDR[1]	1.65
Soy protein isolate[2]	1.40
Sorbitol	14.36
Xylitol	29.02
Ascorbic acid	0.45
Malic acid	0.30
Trisodium citrate	0.75
Algae oil	25.00
Lemon Lime flavor	1.20
Paprika extract	0.08
Witafrol 7420 (AF)	0.08
Stevia	0.10
Water	25.70

[1] Gelagar HDR 800 (B.V. srl, Italy)

[2] Supro 590 (PHH)

The emulsion is prepared according to the general method in Example 1.

Example 4 – Gelled oil-in-water emulsion containing sunflower oil

Ingredient	Wt.%
Agar HDR[1]	1.75
Soy protein isolate[2]	1.50
Sorbitol	16.00
Xylitol	31.43
Ascorbic acid	0.45
Malic acid	0.40
Trisodium citrate	0.90
Sunflower Oil	20.00
Lemon Lime flavor	1.20
Paprika extract	0.08
Witafrol 7420 (AF)	0.08
Stevia	0.10

Water	26.20

[1] Gelagar HDR 800 (B.V. srl, Italy)

[2] Supro 590 (PHH)

The emulsion is prepared according to the general method in Example 1.

Example 5 – Gelled oil-in-water emulsion containing algae oil

Ingredient	**Wt.%**
Agar	1.50
Faba bean protein	1.50
Sorbitol	14.50
Xylitol	29
Malic acid	1
Trisodium citrate	2
Algae oil	25
Flavouring/colouring/sweetening agents/ anti-foaming agent/antioxidant	1.50
Water	24

The emulsion is prepared according to the general method in Example 1.

Example 6 - Gelled oil-in-water emulsion containing algae oil

Ingredient	**Wt.%**
Agar	1.50
Faba bean protein	1.50
Trehalose	21
Sucrose	21
Algae oil	25
Flavouring/colouring/sweetening agents/ anti-foaming agent/antioxidant	1.50
Water and citric acid to pH 4.5	28.5

The emulsion is prepared according to the general method in Example 1 in which trehalose and sucrose are employed as the sugar alcohols. Following homogenisation of the two phases, 50 wt.% citric acid is added until the pH reaches 4.5.

Example 7 – Multi-vitamin supplement

Ingredient	Wt.%
Agar	1.50
Faba bean protein	1.50
Sorbitol	14.5
Xylitol	29
Malic acid	1
Sodium tricitrate	2
Vegetable oil[1]	25
Flavouring/colouring/sweetening agents/ anti-foaming agent/antioxidant	1.50
Water[2]	24

[1]Neutral carrier oil containing 10 mcg Vitamin D and 45 mcg Vitamin K

[2]Water phase containing 2 mcg Vitamin B12

The emulsion is prepared according to the general method in Example 1. Vitamin D and Vitamin K are added to the oil in Step 5 and Vitamin B12 is added to the water phase in Step 6.

Example 8 – Multi-mineral supplement

Ingredient	Wt.%
Agar	1.50
Faba bean protein	1.50
Sorbitol	18.5
Xylitol	31
Malic acid	1
Sodium tricitrate	2
MCT oil	10

Flavouring/colouring/sweetening agents/ anti-foaming agent/antioxidant	1.50
Water[1]	33

[1]Water phase containing 150 mcg iodine, 40 mcg selenium, 20 mg iron and 2.5 mg zinc

The emulsion is prepared according to the general method in Example 1. The minerals are added to the water phase in Step 6.

Example 9 – Calcium supplement

Ingredient	**Wt.%**
Agar	1.50
Faba bean protein	1.50
Sorbitol	14.50
Xylitol	29
Calcium Phosphate Dibasic – $CaHPO_4$	20
Malic acid	1
Sodium tricitrate	2
Vegetable oil[1]	4
Flavouring/colouring/sweetening agents/anti-foaming agent/antioxidant	1.50
Water	25

[1]Vegetable oil containing 400 IU Vitamin D3

The emulsion is prepared according to the general method in Example 1. The calcium phosphate is added to the water phase together with the faba bean protein and sodium tricitrate.

Example 10 – Multivitamin supplement

Ingredient	**Wt.%**	**Amount of Active**
Water	26.00	
Agar HDR	1.75	
Soy protein isolate	1.50	
Sorbitol	15.00	

Xylitol	31.06	
Trisodium citrate dihydrate	0.90	
Sunflower oil	20.00	
Lemon Lime flavour	1.20	
Paprika extract	0.075	
Witrafrol 7420 (AF)	0.075	
Stevia	0.10	
Vitamin C (as Ascorbic Acid)	1.18	15 mg
Vitamin B3 (as Nicotinamide)	0.56	8 mg
Vitamin E (as D-α-Tocopherol)	0.54	5 mg
Vitamin B6 (as Pyridoxine HCl)	0.064	0.7 mg
Vitamin A (as Retinyl palmitate)	0.029	200 mcg
Vitamin D3 (as Cholecaliferol)	0.016	5 mcg
Iodine (as Potassium Iodide)	0.011	30 mcg
Vitamin B12 (as Cyanocobalamin)	0.010	1.25 mcg
Folic acid	0.0087	100 mcg
D-biotin	0.0020	25 mcg

The emulsion is prepared according to the general method in Example 1. The fat soluble vitamins (E, A, D3) are mixed into the oil as in Example 7, and the water soluble vitamins (C, B3, B6, B12, folic acid, D-biotin) as well as iodine are mixed in as the $CaHPO_4$ is mixed in Example 8.

Example 11 - Gelled oil-in-water emulsion containing corn oil

Ingredient	**Wt.%**
Water	33.0
Locust Bean Gum	0.20
Agar HDR	1.80
Soy protein isolate	0.90
Glycerol	34.72
Malic acid	1.10
Trisodium citrate dihydrate	2.20
Corn oil	25.00
Lemon flavour	0.90
Paprika extract	0.075

Witrafrol 7420 (AF)	0.075
Stevia	0.10

The emulsion is prepared according to the general method in Example 1.

Example 12 - Gelled oil-in-water emulsion containing corn oil

Ingredient	**Wt.%**
Water	12.65
Agar HDR	2.00
Soy protein isolate	0.90
Glycerol	55.00
Malic acid	1.10
Trisodium citrate dihydrate	2.20
Corn oil	25.00
Lemon flavour	0.90
Paprika extract	0.075
Witrafrol 7420 (AF)	0.075
Stevia	0.10

The emulsion is prepared according to the general method in Example 1.

Example 13 - packaging

Blister packs:

Prior to setting, the emulsions produced in any of Examples 1-12 may be filled into blister trays made from a metal/plastic laminate or a plastic film over which a plastic/metal foil laminate is heat sealed. Blister trays with pre-formed cavities may be formed from laminated materials such as Tekniflex® Aclar® VA10600 (TekniPlex), Perlalux® (Perlen Packaging), Formpack® (Amcor), and Regula® (Constantia Flexibles).

A liquid emulsion produced in any of Examples 1-12 is filled into blister trays using a syringe and ensuring that the cavities are filled evenly and fully. The blister trays are then flushed with nitrogen for 5-10 seconds, and sealed with a metal/plastic or metal/heat-seal lacquer cover foil by applying a flat iron set at 160°C for 2-4

seconds. The samples are left to cure for 24 hours at room temperature, and submitted to a controlled holding chamber at 40°C for 30 days, 65% RH. On day 5, 10, 15, 20, 25 and 30, samples were withdrawn from the controlled chamber. After 24 hours at room temperature, the blister packs are opened. The amount of residues adhering to the trays and the force required to remove the unit dose from the packs is noted on a scale from 1-9, where 1 indicates no adhesion and very little force required to remove the unit dose ("popping out"), and 9 is full adhesion to the foil and the unit dose needs to be torn from the foil. Each of the laminated materials listed above gives scores of 1, 2 or 3 (mainly 1 or 2) in each test.

Strips:

Prior to setting, the emulsions produced in any of Examples 1-12 may be extruded into individual strips which, once set, are then sealed into individual plastic/metal foil laminate sachets. Alternatively, a single extruded strip, once set, may be cut into individual strips according to need prior to packaging.

Example 14 – Coated gelled emulsions

The set emulsions produced in any of Examples 1-12 may be coated with a sorbitol solution comprising sorbitol (80 wt.%), lemon flavour (0.15 wt.%), yellow colour (0.5 wt.%) and water (ad 100 wt.%). The coating solution may be cured at 99-95°C for 4-5 hours before application. Coating is carried out by dipping or panning at 20-45°C. Several layers of coating material may be added with drying between each layer until the final composite layer is hard.

Alternatively, prior to setting, the liquid emulsion prepared in any of Examples 1-12 may be filled into soft capsule shells. The capsule shell material may typically be a sugar, e.g. sucrose, fructose, maltose, xylitol, maltitol or sorbitol, but may additionally contain hydrocolloid materials such as carrageenan, alginate, pectin, cellulose, modified cellulose, starch, modified starch or gum arabic. The capsule shell may contain further ingredients such as artificial sweeteners, colours, flavours and anti-oxidants.

Example 15 – Effect of different surfactants on dynamic storage modulus (G' max)

Gelled oil-in-water emulsions containing 2.5 wt.% agar were prepared using soy bean protein, pea protein (Nutralys F85M), propylene glycol alginate (degree of esterification: 84%) (PG alginate), Tween 80 and LACTEM as surfactants. Gelled oil-in-water emulsions having agar concentrations ranging from 0.75 to 3 wt.% were also produced using soy bean protein. All formulations were tested for their rheology characteristics.

The following formulation containing 2.5 wt.% agar as gelling agent was prepared according to the general protocol in Example 1:

Ingredients	**Wt.%**	**Wt. (in 50g)**
Water	24.36	12.18
Agar HDR	2.50	1.25
Xylitol	29.00	14.5
Sorbitol	14.95	7.475
Surfactant[1]	0.75	0.375
Malic acid	1.16	0.58
Trisodium citrate dihydrate	2.28	1.14
Corn oil	25.00	12.5
Total	**100.00**	**50**

[1]Soy bean protein, pea protein, PG alginate, Tween 80 or LACTEM

Formulations with agar concentrations in the range 0.75 to 3.0 wt.% were also prepared using soy bean protein as a surfactant. Any change in agar concentration was compensated by an equivalent change in sorbitol content.

Soy bean protein, pea protein and PG alginate are high molecular weight surfactants (i.e. "macromolecular"), whereas Tween 80 and LACTEM are low molecular weight surfactants. Tween 80 is a polysorbate surfactant derived from polyethoxylated sorbitan and oleic acid. LACTEM consists of lactic acid esters of mono and diglycerides.

Stable emulsions could not be prepared with Tween 80 or LACTEM. In respect of all other formulations, the dynamic storage modulus (G' max) was measured according to the small scale deformation test described herein. The results are shown in Figure 1. With increasing agar concentration it could be observed that the small scale deformation modulus of the solid emulsion increased when using the macromolecular surfactants (soy/pea protein and PG alginate). Although not wishing to be bound by theory, this is believed to be due to a friction layer around the droplets created by an uneven distribution of large molecules ("hairy"), which provides a "semi-active filler" effect. This effect increases with increasing agar concentration. All macromolecular surfactants gave stable emulsions.

Example 16 – Press-testing

The following formulation containing 2.5 wt.% agar as gelling agent was prepared according to the general protocol in Example 1:

Ingredient	**Wt.%**	**Wt. (in 50g)**
Water	24.36	12.18
Agar HDR	2.50	1.25
Xylitol	29.00	14.5
Sorbitol	14.95	7.475
Soy protein	0.75	0.375
Malic acid	1.16	0.58
Trisodium citrate dihydrate	2.28	1.14
Corn oil	25.00	12.5
Total	**100.00**	**50**

Formulations with agar concentrations in the range 0.25 to 3.0 wt.% were also prepared. The change in agar concentration was compensated by an equivalent change in sorbitol content.

Whilst still liquid, the emulsions (1 ml) were poured into standard non-stick blister foil packs and sealed with a flat iron at 150°C. After 24 hours at room temperature the agar emulsions had solidified and press-testing was carried out to evaluate the minimum agar concentration at which the gelled tablets could be squeezed out of the blister forms without breaking. The lowest agar concentration that could

withstand this without breaking into pieces was 0.75 wt.%. This corresponds to a G' max of around 15 kPa. The highest acceptable agar concentration was found to be 2.5 wt.% based on visual observations where the solution before setting became very thick. That corresponded to a measured viscosity at 55°C of 60 Pa.s at a shear rate of 1/s and 23 Pa.s at a shear rate of 10/s.

Example 17 – Effect of different surfactants

Tests were carried out to assess the impact of the molecular weight of the surfactant. As reported in Example 15, the high molecular weight surfactants - soy and pea proteins and propylene glycol alginate (PG alginate) - provided a stable emulsion having a high dynamic storage modulus (G' max). This was attributed to formation of a 'hairy' droplet surface formation acting as a semi-active filler through increased friction. In the following series of experiments, hydroxy propyl methyl cellulose (HPMC) materials having an identical degree of substitution but with varying weight average molecular weights were tested. HPMC materials were obtained from Shin-Etsu Tylose GmbH having the following properties:

HPMC Material	**Weight Average Molecular Weight, Mw (kDa)[1]**
Metolose SB-4	24
Metolose 90SH-100SR	94
Metolose 90SH-15000SR	435

[1] Weight average molecular weight was determined based on a conversion factor from viscosity to Mw provided by the manufacturer ($Mw = 40000 \times \log \eta + 880 \times (\log \eta)^4$ wherein Mw = weight-average molecular weight; η = solution viscosity).

The basic formulation used in this experimental series was as follows:

Ingredient	**Wt.%**	**Wt. (in 50g)**
Water	24.36	12.18
Agar[1]	1.00	0.5
Xylitol	29.00	14.5
Sorbitol	16.45	8.225
Surfactant	0.75	0.375
Malic acid	1.16	0.58

Trisodium citrate dihydrate	2.28	1.14
Corn oil	25.00	12.5
Total	**100.00**	**50**

[1]B&V Gelagar HDR 800 (B.V. srl, Italy)

Preparation of oil-in-water emulsions:

1. Agar and sugar alcohols were mixed as a dry powder and added to a bottle with the correct amount of water. The bottle was put in a water bath at 90°C with magnetic stirring for 30 minutes.
2. The bottle was transferred to a water bath at 50°C, equilibrated for 15 minutes. This was the stage where HPMC surfactant was added, and these mixtures were left for 30 minutes to allow the HPMC to dissolve.
3. The pre-heated (50°C) corn oil was added and homogenisation was carried out using an Ultra-Turrax for 5 minutes.
4. The resulting emulsion was put back at 55°C for 10 minutes before rheological examination.
5. Rheological experiments were carried out applying a Kinexus Ultra+ Rheometer equipped with a C 4/40 measuring geometry. Strain 0.1%, a 1 Hz frequency, a temperature gradient from 50 to 20°C and a holding time of 20 minutes at 20°C was applied before a G' value for the different systems was recorded.

The results are presented in Table 1:

Table 1

Surfactant	**Average Mw (kDa)**	**G' max (kPa)**
Metolose SB-4	24	9.9
Metolose 90SH-100SR	94	20.2
Metolose 90SH-15000SR	435	19.5

An increase in weight average molecular weight from 24 to 94 kDa led to a doubling of the G' max value when using HPMC as a macromolecular surfactant. No further increase with molecular weight was observed beyond that. It was observed by visual inspection that the lowest Mw HPMC surfactant gave higher syneresis (release of water phase) than the higher Mw ones. All HPMC formulations were able to retain the oil when exposed to mechanical stress. Higher Mw surfactants

thus give additional benefits other than increased gel strength, i.e. less syneresis and better retention of oil.

Example 18 – Tests on gelled oil-in-water compositions containing agar and gum arabic to show the effect of increasing agar concentration

50 g formulations containing the following ingredients were prepared using methodology analogous to that in Example 1.

	Example 18A	**Example 18B**
Ingredients	**Wt.%**	**Wt.%**
Agar[1]	1.13	1.50
Gum Arabic	1.00	1.00
Water	24.88	24.5
Xylitol	29	29
Sorbitol	14.5	14.5
Sodium tricitrate	2	2
Malic acid	1	1
Corn oil	25	25
Faba bean protein[2]	1.5	1.5
Total	**100.0**	**100.0**

[1] Gelagar HDR 800 (B.V. srl, Italy)

[2] Vestkorn Faba Protein F65X (Vestkorn A/S, Denmark)

Droplet sizes and size distributions were measured using a Malvern Mastersizer 3000 (Worcestershire, UK) connected to a Hydro MV, wet dispersion unit (Malvern,Worcestershire, UK). Analysis of the data was performed using the manufacturer's software (Mastersizer 3000, v1.0.1). Testing was carried out by dissolving and diluting the gelled emulsion in a 10% (v/v) HCl solution (1:100) at 50°C. The refractive index of water and corn oil was set to 1.33 (solvent) and 1.47 (dispersed phase), respectively, and the absorption index of the dispersed droplets set to 0.01. To avoid multiple scattering or low intensity of the scattered light, each dissolved emulsion was added to the dispersion unit (containing ~125 mL water), until an obscuration of approximately 10% was obtained. Droplet size distributions for the different emulsions are shown in Table 2.

Table 2

	D [4;3]	D [3;2]	Dx(10)	Dx (50)	Dx (90)
Example 18A	8.75	3.59	1.66	7.56	16.98
Example 18B	13.64	4.756	2.908	12.4	25.64

Dynamic storage modulus (G' max) was measured according to the small scale deformation test described herein. The results in Figure 2 show the shear modulus (elastic component) of the resulting emulsion as a function of temperature and time. G' max for the formulation containing 1.13 wt.% agar (Example 18A) was 15,650 Pa, whereas that for the formulation containing 1.50 wt.% agar (Example 18B) was 21,220 Pa. This confirms an increase in the strength of the gel with increasing agar concentration. Both formulations exhibited a 'solid-like' nature over a wide temperature range.

Example 19 – Tests on gelled oil-in-water compositions containing agar with and without gum arabic

50 g formulations containing the following ingredients were prepared using methodology analogous to that in Example 1.

	Example 19A	Example 19B
Ingredients	**Wt.%**	**Wt.%**
Agar[1]	1.50	1.50
Gum Arabic	1.00	0
Water	24.5	24.5
Xylitol	29	29.5
Sorbitol	14.5	15
Sodium tricitrate	2	2
Malic acid	1	1
Corn oil	25	25
Faba bean protein[2]	1.5	1.5
Total	**100.0**	**100.0**

[1] Gelagar HDR 800 (B.V. srl, Italy)

[2] Supplied by Vestkorn

Droplet sizes and size distributions were measured as described in Example 18. The results are shown in Table 3.

Table 3

	D [4;3]	D [3;2]	Dx (10)	Dx (50)	Dx (90)
Example 19A	14.76	5.248	3.196	13.16	28.14
Example 19B	24.12	7.55	6.248	22.3	44.18

Texture analysis was carried out using a standard TPA test as described herein. The results are shown in Table 4 and in Figure 3.

Table 4

	Ex. 19A (7 samples)	**Ex. 19B** (10 samples)
Hardness (g)	312.9 ± 8.9	413.5 ± 7.7
Adhesiveness (g.sec)	-45.5 ± 11.7	-42.6 ± 9.8
Resilence (%)	59.6 ± 1.5	62.2 ± 0.5
Cohesion	0.8 ± 0.0	0.8 ± 0.0
Springiness (%)	95.4 ± 1.1	96.9 ± 0.8
Gumminess	257.8 ± 7.4	349.8 ± 7.9
Chewiness	245.8 ± 6.6	338.8 ± 9.2

In general, the presence of gum arabic was found to result in gelled emulsions having smaller droplet sizes and which are slightly softer.

Example 20 – Droplet size and size distribution

50 g formulations containing the following ingredients were prepared using methodology analogous to that in Example 1.

	Example 20 Wt.%	Temperature (°C)	Mixing time (min)
Agar	1.5	90	30
Xylitol	29		
Sorbitol	14.5		
Stevia	0.1		
Water	24.125		
Malic acid	1	65	10
Faba bean protein (Vestkorn)	1.5		45
Trisodium Citrate	2		10

Paprika extract	0.075	50	2
Lemon flavor	1.2		
Algae oil	25		
Total	**100**		Homogenization 7 min at speed 5

Droplet sizes and size distributions were measured as described in Example 18. Rheological analysis was carried out according to the small scale deformation test described herein. In accordance with standard rheological measurement methods, Tg and Tm were determined when the phase angle dropped below or went above 45° under the given temperature gradient, strain and frequency. The results are provided in Table 5 and in Figure 4.

Table 5

	D [4;3]	D [3;2]	Dx (10)	Dx (50)	Dx (90)	G' max (kPa)	Tg (°C)	Tm (°C)
Ex. 20	22.08	7.976	8.32	20.76	38.34	15	39.5	88.7

Example 21 - Droplet size and size distribution

50 g formulations containing the following ingredients were prepared using methodology analogous to that in Example 1.

	Example 21A Wt.%	**Example 21B Wt.%**	Temperature (°C)	Mixing time (min)
Agar	1.75	2	90	30
Xylitol	28.875	28.75		
Sorbitol	14.375	14.25		
Stevia	0.1	0.1		
Water	24.125	24.125		
Malic acid	1	1	65	10
Faba bean protein (Vestkorn)	1.5	1.5		45
Trisodium citrate	2	2		10
Paprika extract	0.075	0.075	50	2
Lemon flavor	1.2	1.2		
Algae oil	25	25		
Total	**100**	**100**		Homogenization 7 min at speed 5

Droplet sizes and size distributions were measured as described in Example 18. The results are provided in Table 6.

Table 6

	D [4;3]	D [3;2]	Dx (10)	Dx (50)	Dx (90)
Example 21A	23.88	7.81	5.224	22.7	43
Example 21B	15.34	7.21	7.862	14.98	24.14

Example 22 - large scale deformation of gelled oil-in-water emulsion

Experiments were carried out to compare the large scale deformation of agar-based emulsions prepared according to Examples 19A and 19B and that of pure aqueous agar gels. Pure aqueous agar gels were prepared by mixing agar (2 wt.%) and Milli-Q-water (MQ- H_2O) at 90°C. The mixture was cooled down to ambient temperature for further characterization of the gels.

Tests were carried out according to the large scale deformation method described herein. The results are shown in Figure 5 and in Table 7.

Table 7

	Young's modulus (N/m^2)	Max stress (g)	Strain at failure (%)
1.5% pure agar gel (10 samples)	29354.2 ± 2444.8	495.2 ± 42.1	26.8 ± 0.9
Example 19A (6 samples)	48017.8 ± 1749.5	340.4 ± 17.4	25.7 ± 1.2
Example 19B (6 samples)	64100.9 ± 5258.2	423.4 ± 25.5	25.1 ± 1.2

The Young's modulus (or initial slope of the force/deformation curve in this context) is somewhat higher for the agar-based gelled emulsions according to the invention which means these provide more resistance at very low deformation. However, these compositions conserved much more structure after failure (at around 25% strain) compared to the pure aqueous agar gels. This means that the agar-based gelled emulsions according to the invention will not fracture in the mouth and thus provide a more attractive chewing experience.

Example 23 – Syneresis Tests

Experiments were carried out to compare the syneresis of the agar-based emulsions according to Examples 20 and 21B (containing 1.5 wt.% and 2.0 wt.% agar, respectively) and that of a pure aqueous agar gel. The pure aqueous agar gel was made according to the same method as in Example 22. Each gel was subjected to a freeze-thaw cycle and the average weight loss was measured as described in the syneresis tests described herein. Results are shown in Table 8.

Table 8

	Average weight loss (%)	**Standard deviation**
2.0 % pure agar gel (3 samples)	51.0	5.7
Example 20 **Agar content: 1.5 wt.%** (6 samples)	2.48	0.45
Example 21B **Agar content: 2.0 wt.%** (3 samples)	2.24	0.13

The pure agar gel lost over 50% of its original water content following the freeze-thaw cycle which indicates significant syneresis. The water loss for the gelled oil-in-water emulsions according to the invention is approx. 20-fold lower. The content of the agar did not significantly influence the extent of syneresis and both gelled emulsions according to the invention showed acceptable syneretic properties

Example 24 – Effect of glycerol

The aqueous solvent for use in producing the formulations according to the invention may be modified by the incorporation of glycerol. Aqueous agar gels were produced in which water was successively exchanged with glycerol in order to determine the relative changes in the properties of the aqueous gel. The glycerol concentration was varied from 0 to 90 wt.% and water activity was measured as described herein. The results are shown in Figure 6. At a 50:50 mixture of water:glycerol a water activity of below 0.8 was achieved. At this water activity, microbial growth is prevented. By using glycerol instead of water there could be a reduced need for sugar alcohols to reduce water activity and to obtain a product which is stable to microbial degradation.

G' max and gelling temperature were also measured for the different gels according to the the small scale deformation method described herein. With an increasing content of glycerol an increase in dynamic storage modulus was observed up to 50% inclusion of glycerol. The gelling temperature started to drop markedly around the same glycerol concentration. Large scale deformation and penetration showed an increase in resistance with increasing glycerol contents up to 50%. This is more or less in line with the small scale deformation results (G'). At the same time an increase of up to around 30% in compression distance before break was recorded at low and medium glycerol contents. This was also confirmed in a chewing test where the glycerol containing gels were perceived as being more "gelatin-like" than without.

Example 25 – Testing of gelled oil-in-water compositions with higher oil content and comparison with gelatin-based oil-in-water compositions.

A gelled oil-in-water composition containing agar and faba bean protein and 40 wt.% oil was prepared according to Example 21B, but without paprika, lemon or stevia. The oil was added gradually, first up to 25 wt.%, then up to 30 wt.%, then 35 wt.%. In each step, the oil was first mixed in with a magnet or spatula before homogensiation using an ultra turrax machine. The final 5 wt.% oil (up to 40 wt.%) was incorporated by pure shaking due to very high viscosity at which the ultra turrax could not mix properly. A pure agar gel (without oil) was made as a comparison. An aqueous gelatin gel was also made for comparison using 260 Bloom type B bovine gelatin, 6.67 wt% in water. This was gelled at 4°C overnight in cylinders and fully equilibrated to room temperature before texture measurements.

Large scale deformation measurements were carried out as described herein. The results for the agar-based compositions (without oil and with 40 wt.% oil) are provided in Figure 7 and those for the gelatin gels are shown in Figure 8. These are also provided in Table 9

Table 9

	Agar gel - no oil	**Agar gelled emulsion - 40 wt.% oil**	**Gelatin gel**
Force at break (g)	1734 ± 25	734 ± 21	1460 ± 139

Strain at break	29.3 ± 0.3	30 ± 1	73 ± 1
Gradient (g/mm)	196 ± 3	80 ± 2	9.4 ± 0
Young's modulus (kPa)	215 ± 4	88 ± 2	10.3 ± 0.3

The Young's modulus becomes more comparable to that of the gelatin gels in the agar gels containing 40 wt.% oil compared to without oil. The overall maximum gel strength decreases with the presence of oil, but shows the same large scale preservation of structure (relatively more so than without the oil present, although the sugar alcohols alone contribute quite significantly to the preserved structure at high strains).

Gelatin gels typically fail at much higher strain and show high resistance at high deformation, which compares to the deformation of chewing. It is the particular conservation of structure at strains above e.g. 40% that makes the agar gel formulations with oil and macromolecular surfactants according to the invention more comparable to gelatin.

Droplet size measurements were are also carried out in respect of the agar-based emulsions having different oil contents. The emulsions were slightly flocculated after dilution in water and SDS was added to deflocculate before droplet size measurements. Before measurement, successful deflocculation was confirmed with optical microscopy. Droplet sizes of the emulsions are given in Table 10.

Table 10

	D [4;3]	D [3;2]	Dx (10)	Dx (50)	Dx (90)
faba 30 wt.% oil initial	13.5	5.16	2.99	11.5	25.2
faba 35 wt.% oil initial	11.8	4.5	2.36	9.07	18.7
faba 40 wt.% oil initial	8.92	4.23	2.71	7.57	15.1
faba 40 wt.% oil after 3 hours at 55°C	9.78	4.46	3.03	7.95	17.2
faba 40 wt.% oil after 20 hours at 55°C	16.8	6.43	4.25	13.6	33.8

Claims:

1. An orally administrable, gelled oil-in-water emulsion which is a self-supporting, viscoelastic solid having a gelled aqueous phase comprising a gelling agent which is agar, and wherein said emulsion is stabilised by a surfactant which is a plant-based protein, plant-based polysaccharide or derivative thereof.

2. An orally administrable, gelled oil-in-water emulsion as claimed in claim 1, wherein said surfactant is a plant-based protein or derivative thereof.

3. An orally administrable, gelled oil-in-water emulsion as claimed in claim 2, wherein said protein is obtained from a plant in the legume family, preferably from a pea or bean.

4. An orally administrable, gelled oil-in-water emulsion as claimed in claim 3, wherein said protein is faba bean protein or soy bean protein.

5. An orally administrable, gelled oil-in-water emulsion as claimed in claim 1, wherein said surfactant is a plant-based polysaccharide or derivative thereof.

6. An orally administrable, gelled oil-in-water emulsion as claimed in claim 5, wherein said surfactant is a hydrophobically-modified polysaccharide.

7. An orally administrable, gelled oil-in-water emulsion as claimed in claim 5 or claim 6, wherein said surfactant is a cellulose or a cellulose derivative, a starch or a starch derivative, or propylene glycol alginate.

8. An orally administrable, gelled oil-in-water emulsion as claimed in any one of the preceding claims which contains agar at concentration from about 0.1 to about 5 wt.% based on the total weight of the emulsion.

9. An orally administrable, gelled oil-in-water emulsion as claimed in any one of the preceding claims which further contains glycerol.

10. An orally administrable, gelled oil-in-water emulsion as claimed in any one of the preceding claims, wherein said aqueous phase constitutes from 50 to 95 wt.%, preferably from 55 to 90 wt.%, for example from 60 to 85 wt.%, from 70 to 85 wt.%, or from 75 to 80 wt.% of the emulsion.

11. An orally administrable, gelled oil-in-water emulsion as claimed in any one of the preceding claims, wherein the aqueous phase further comprises one or more bulking agents, for example sugar alcohols or sugars.

12. An orally administrable, gelled oil-in-water emulsion as claimed in claim 11, wherein said bulking agents are present at a concentration of from 45 to 70 wt.%, preferably 50 to 65 wt.%, e.g. 55 to 60 wt.%, based on the aqueous phase.

13. An orally administrable, gelled oil-in-water emulsion as claimed in any one of the preceding claims having an oil phase which comprises one or more physiologically tolerable lipids derived from rapeseed oil, sunflower oil, corn oil, olive oil, sesame oil, palm kernel oil, coconut oil, a nut oil, algae oil or hemp oil.

14. An orally administrable, gelled oil-in-water emulsion as claimed in any one of the preceding claims having an oil phase which constitutes from 5 to 50 wt.%, preferably from 10 to 45 wt.%, for example from 15 to 40 wt.%, from 15 to 30 wt.% or from 20 to 25 wt.%. of the emulsion.

15. An orally administrable, gelled oil-in-water emulsion as claimed in any one of the preceding claims which further comprises at least one pharmaceutically active agent.

16. An orally administrable, gelled oil-in-water emulsion as claimed in any one of the preceding claims which further comprises at least one nutraceutically active agent.

17. An orally administrable, gelled oil-in-water emulsion as claimed in claim 16, wherein said nutraceutically active agent is a vitamin or a mineral.

18. An orally administrable, gelled oil-in-water emulsion as claimed in any one of the preceding claims which is provided in unit dose form.

19. An orally administrable, gelled oil-in-water emulsion as claimed in claim 18, wherein said unit dose form is uncoated.

20. A package comprising an air-tight and light-tight compartment containing one dose unit of the gelled oil-in-water emulsion as claimed in claim 18 or claim 19.

21. A package as claimed in claim 20 which is a blister pack formed from a material which is not coated with a release agent.

22. A method for the preparation of an orally administrable, gelled oil-in-water emulsion as claimed in any one of claims 1 to 19, said method comprising the steps of: forming an oil phase which comprises one or more physiologically tolerable lipids; forming an aqueous phase comprising a gelling agent which is agar; combining said oil phase and said aqueous phase to form an oil-in-water emulsion in the presence of a surfactant which is a plant-based protein, plant-based polysaccharide or derivative thereof; and allowing said emulsion to gel.

23. A gelled oil-in-water emulsion as claimed in any one of claims 1 to 19 for oral use as a medicament or for oral use in therapy.

24. A gelled oil-in-water emulsion as claimed in any one of claims 1 to 19 which contains at least one pharmaceutically active component for oral use in the treatment of a condition responsive to said pharmaceutically active component.

25. Use of a gelled oil-in-water emulsion as claimed in any one of claims 1 to 19 as a nutraceutical.

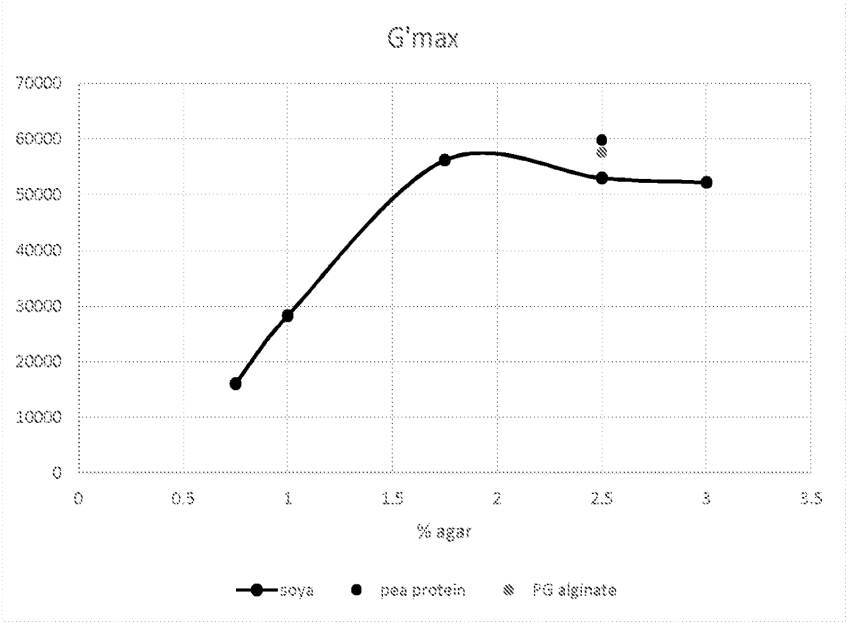


Fig. 1

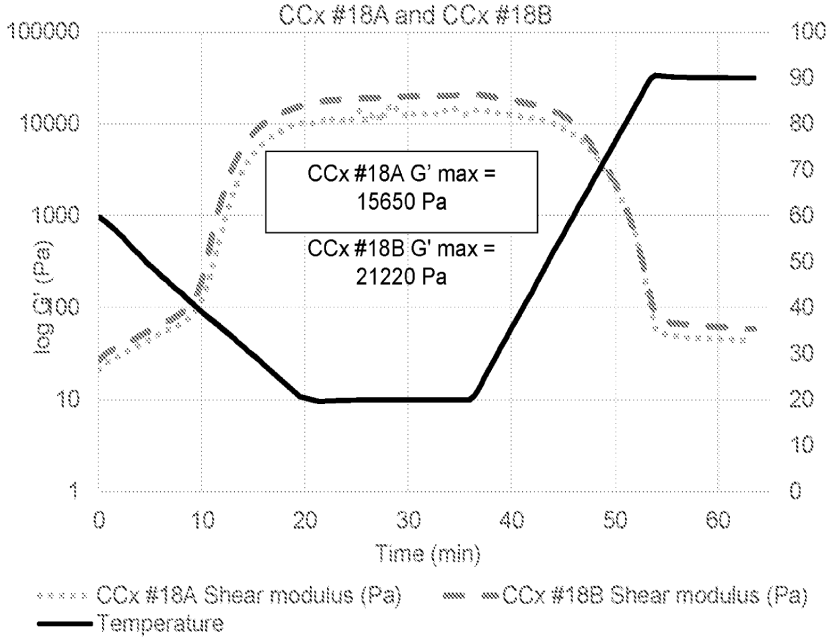
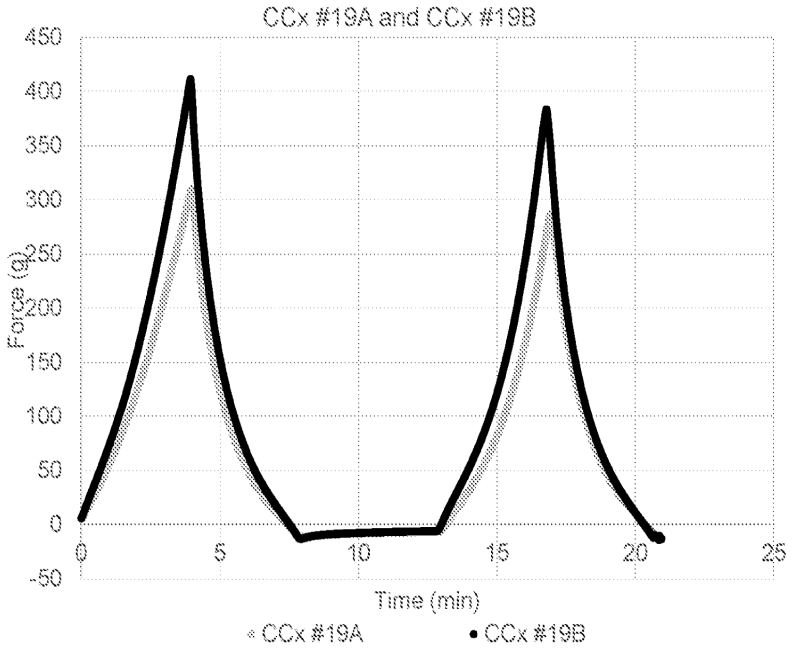


Fig. 2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5
10
15
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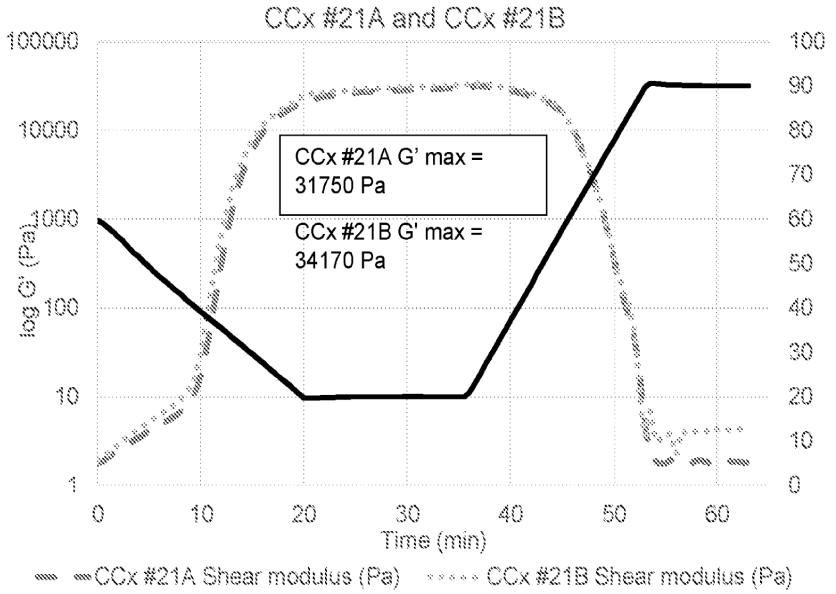
Time (min)
CCx #19A
CCx #19B

Fig. 3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10
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CCx #21B G' max = 34170 Pa
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40
50
60
Time (min)
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CCx #21B Shear modulus (Pa)

Fig. 4

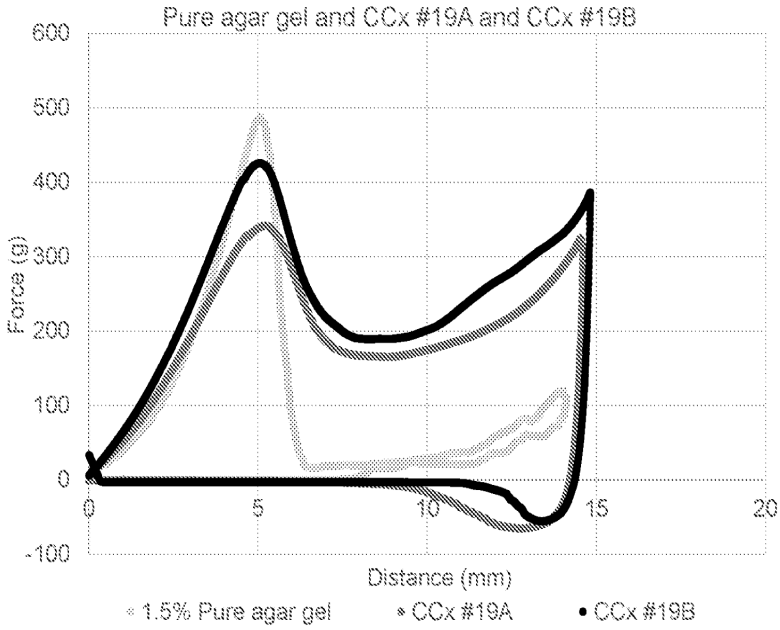
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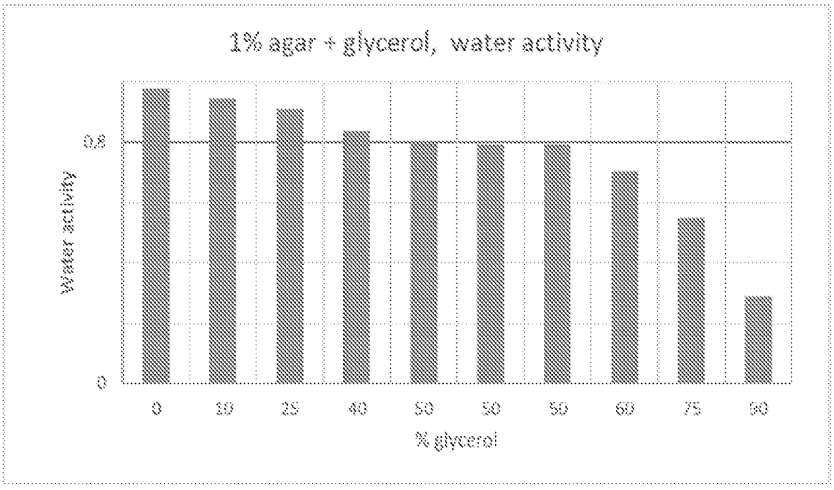
Fig. 5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50
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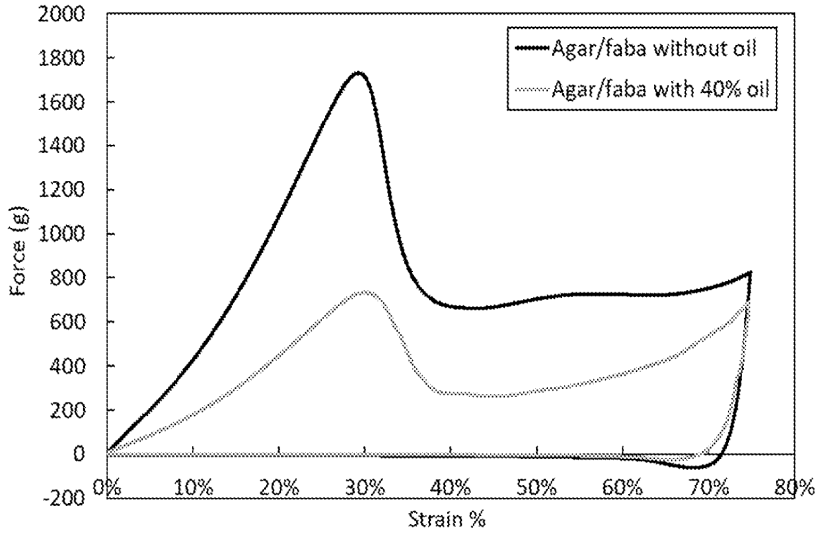
% glycerol

Fig. 6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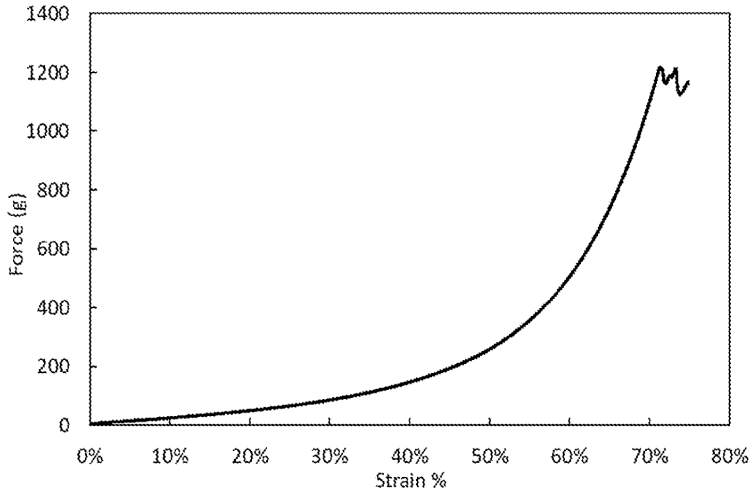
10%
20%
30%
40%
50%
60%
70%
80%

Fig. 7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Strain %
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1000
800
600
400
200
0
0%
10%
20%
30%
40%
50%
60%
70%
80%

Fig. 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2022/050962

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/10 A61K47/26 A61K47/36 A61K47/38 A61K47/42
A61K47/44 A61K9/00 A23L2/00 A61K9/107
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	EP 3 437 488 A1 (RIKEN VITAMIN CO [JP]) 6 February 2019 (2019-02-06) table 4 -----	1-3,11, 15-25
X	RO 133 469 A0 (UNIV DUNAREA DE JOS GALATI [RO]) 30 July 2019 (2019-07-30) example 1 -----	1,5-7, 13, 16-22,25
X	AU 2019 203 259 A1 (DOMALINA PTY LTD [AU]) 28 November 2019 (2019-11-28) examples 2, 4, 5 -----	1,5, 15-25
	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 July 2022	28/07/2022

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Frelichowska, J

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X,P	WO 2021/099792 A1 (VITUX GROUP AS [NO]; GOLDING LOUISE [GB]) 27 May 2021 (2021-05-27) example 17 -----	1,5,11, 13-16, 18-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2022/050962

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		ES 2210850 T3	01-07-2004
		ID 25929 A	09-11-2000
		JP 3648597 B2	18-05-2005
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