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Protein aerogel
micro particles

mixed with fish oil
(state of the art)

impregnated
with fish oil from
supercritical CO₂

Encapsulation of fish oil in protein aerogel micro-particles

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1. Introduction

One objective of the food industry is to increase the shelf-life of sensitive nutrients and to control their release in the human digestive system. Encapsulation enables the protection of sensitive ingredients from adverse conditions like oxygen, moisture, temperature or light and offers the possibility to mask undesirable tastes and odors of bioactive functional food additives (Tavares et al., 2014). One way of encapsulation is the concept of aerogel manufacture and loading. Aerogels can be defined as dry, open mesoporous, coherent networks of inorganic or organic solid material formed by gelation and subsequent supercritical drying. A decisive point of difference of aerogels in comparison to other drying methods is the retention of shape and inner structure during supercritical drying. The inner specific surface area of aerogels is therefore extremely high and available for adsorption, while, in addition, the high inner porosity is finely structured offering a large inner volume for capillary condensation of sensitive material. The filled pores of the aerogel structure should protect sensitive substances from contact with air and oxidation. Additionally, the mesoporous structure itself acts as transport residence for air and oxidation products.

Aerogels based on natural biopolymers, like polysaccharides or proteins, are novel carrier materials for pharmaceutical products due to their biocompatibility and biodegradability

(Mehling et al., 2009). Additionally, they are safe, edible, renewable and sustainable and thus also applicable in food systems (Mikkonen et al., 2013). Their open mesoporous structure results in extraordinary properties like large specific BET (Brunauer-Emmet-Teller)-surface areas and high porosities building the basis for a carrier material (Mikkonen et al., 2013). Aerogels made from natural polymers, mainly from polysaccharides in form of micro-particles, were investigated in several studies as transport matrices for pharmaceuticals (Alnaief et al., 2011; Baldino et al., 2016; Betz et al., 2012; Cicco et al., 2016; García-González et al., 2011; García-González et al., 2015; Gonçalves et al., 2016; Haimer et al., 2010; Lovskaya et al., 2015; Maleki et al., 2016; Marin et al., 2014; Mehling et al., 2009; Mustapa et al., 2018; Obaidat et al., 2018; Salgado et al., 2017; Stergar and Maver, 2016; Ulker and Erkey, 2014; Veres et al., 2018; Zhao et al., 2018). For food applications, aerogel micro-particles are promising since their integration into food matrices does not change the food structure significantly. The consumer acceptance of food additives encapsulated in aerogel micro-particles is assumed to be higher than of those being encapsulated in aerogel monoliths.

In principal, the production of biopolymer-based aerogels consists of three steps: Gel formation in aqueous solution, solvent exchange from water to an organic solvent (e.g. ethanol) followed by the supercritical CO₂-drying of the gel matrix. The integration of a valuable compound into an aerogel matrix can be conducted by an addition in different aerogel production steps depending on the valuable compound's affinity to the used solvents (water, organic solvent, supercritical CO₂) (Comin et al., 2012; Maleki et al., 2016; Ulker and Erkey, 2014):

- a) before gelation,
- b) during solvent exchange,
- c) during supercritical drying
- d) after supercritical drying.

The addition after gel formation (possibilities b) – d)) is interesting for the food industry since a separate production of the transport matrix from its loading enables gelation at high temperatures or extreme pH-values without degradation of the sensitive valuable compound. After loading the matrix, an additional coating layer can be applied onto the aerogels as additional protection (Alnaief et al., 2010; Alnaief et al., 2012; Goslinska et al., 2019; Kleemann et al., 2018). The interest in biopolymer-based aerogels and cryogels (obtained by freeze-drying) as transport matrices for food applications becomes obvious when considering the increasing number of studies in the last few years covering this topic (Ahmadi et al., 2016; Comin et al., 2012; Haimer et al., 2010; Marco and Reverchon, 2017; Pantić et al., 2016b; Pantić et al., 2016a). These studies show that biopolymer-based aerogels can be

loaded with significant amounts of oil or vitamin by various loading techniques. Mostly, polysaccharides were used as matrix material in these studies or conventional freeze drying was applied to convert a hydrogel into a dried matrix. This study uses natural proteins as matrix material in combination with supercritical CO₂-drying of the protein gel matrix. Such monolithic protein-based aerogels were tested already in first studies as carrier matrices for drug release (Betz et al., 2012; Marin et al., 2014) but not for food applications. In previous studies we developed protein aerogels in the form of micro-particles consisting of natural proteins obtained from milk and egg white designed for food systems (Betz et al., 2012; Kleemann et al., 2018; Selmer et al., 2015). Additionally, we successfully tested the coating of these protein-based aerogel micro-particles (Goslinska et al., 2019). In the present paper, we evaluate the loading of the previously developed protein aerogel particles made of structurally different protein materials with exemplary core materials of interest for application in food systems. Aerogel micro-particles produced from the protein sources whey protein isolate (WPI), sodium caseinate (NaCas) and egg white protein (EWP) were utilized. These proteins have different molecular structures, different reactive sites for crosslinking and the mechanisms of gel formation are partially different. Therefore, the precursor hydrogels are different as well as their resulting supercritically dried aerogel structures (Kleemann et al., 2018; Selmer et al., 2015). We expect that the different aerogel structures show different loading capacities. Fish oil was selected as exemplary model additive. The purpose was to protect it against oxidation. Fish oil is rich in essential unsaturated ω -3-fatty acids, which have positive effects on the human health, but are sensitive to oxidation. The oxidation products of the ω -3-fatty acids produce rancid odors and flavors (Miyashita et al., 2018). The possibilities of protecting the core material against oxidation and masking potential off-flavors make fish oil encapsulation technologies highly interesting for the food industry. (Bakry et al., 2016; Encina et al., 2016). The aim of this study is to achieve high specific loadings of the chosen biological functional model substances onto the aerogel particles using different loading techniques to evaluate more broadly the potential of protein aerogels as a carrier material in food systems.

2. Materials and methods

2.1. Materials

Pasteurized egg white was provided from Ovobest Eiprodukte GmbH & Co. KG (Neuenkirchen-Vörden, Germany). Whey protein isolate with a protein content of 94% (w/w) was purchased from Davigo Foods International Inc. (Eden Prairie, USA). Sodium caseinate N94 with a protein content of 90% (w/w) was obtained from Molkereigesellschaft Lauingen mbH (Lauingen, Germany). Activa MP Transglutaminase formulation with a specified activity of 100 U/g powder was provided by Ajinomoto Foods Europe S.A.S. (Paris, France). Pure

Sunflower oil was purchased from a local store. Polyglycerin-Polyricinoleat (PGPR) 4150 was provided by Palsgaard (Juelsminde, Denmark) and fish oil from Fresenius Kabi AG (Bad Homburg v. d. Höhe, Germany). Marine Oil FAME Mix cat.: 35066 from Restek GmbH (Bad Homburg v. d. Höhe, Germany) was utilized as standard for fatty acid methyl esters. Hexane (> 95% purity) was obtained from Merck KGaA (Darmstadt, Germany) and food grade carbon dioxide from Praxair Technology, Inc. (Düsseldorf, Germany). All other chemicals were purchased from Carl Roth (Karlsruhe, Germany) or Merck KGaA (Darmstadt, Germany) and were of analytical grade.

2.2. Methods

2.2.1. Protein aerogel micro-particles

The utilized spherical protein aerogel micro-particles were produced via emulsion gelation method followed by supercritical drying according to KLEEMANN ET AL. (Kleemann et al., 2018). Two sorts of WPI-aerogel particles with different pore structures and mean particle sizes were produced: During preparation of the small hydrogel particles deionized water was utilized as washing media (Kleemann et al., 2018) in case of WPI I particles and 10 mM CaCl_2 -solution was utilized in case of WPI II particles. Further, EWP- and NaCas-aerogel particles were tested. The aerogel particles' appearance under the electron microscope is shown in Figure 1. Their properties are presented in Table 1.

Table 1: Properties of the protein aerogel particles determined according to the methods described in SELMER ET AL. (Selmer et al., 2015).

Property	Unit	WPI I	WPI II	EWP	NaCas
BET-surface area	m^2/g	354 ± 37	154 ± 15	232 ± 12	48 ± 5
Pore volume (BJH)	cm^3/g	1.55 ± 0.11	0.33 ± 0.13	2.28 ± 0.18	0.28 ± 0.13
Mean pore diameter*	nm	14.0	7.1	41.7	13.4
Mean particle size*	μm	66.6 ± 1.6	47.3 ± 1.1	32.7 ± 0.4	40.9 ± 0.0
Bulk density**	kg/m^3	241 ± 3	-	179 ± 2	443 ± 1

*The pore and particle size distributions are presented in the supplementary information. The particle size distribution (of 7,000 to 10,000 particles per sample) was measured twice using a Camsizer XT with X-Jet module (Retsch GmbH, Haan, Germany).

**The bulk density was calculated from measured weight (± 0.0001 g) and volume (adjusted to 1 mL after three taps) of the aerogel particles (triplicate measurements).

2.2.2. Static adsorption of fish oil from supercritical CO_2 on protein aerogel micro-particles

Two different sets of static adsorption experiments were conducted. The first set of static adsorption experiments was used to investigate the thermodynamic equilibrium. Fish oil saturated supercritical CO₂ was utilized in all experiments. The thermodynamic equilibrium was measured by simultaneous triple measurements in three (of the four) different high-pressure autoclaves being identical in construction (Figure 2). 3 mL of fish oil were placed with a syringe at the bottom of each of the 40 mL autoclaves. 0.6 g of the WPI- or NaCas-aerogel particles or rather 0.3 g of the EWP-aerogel particles were packed in a filter paper which was rolled up and clamped in each autoclave above the fish oil without any contact. The autoclaves were closed and preheated. The CO₂ was delivered from a CO₂ tank, liquified by cooling and afterwards pumped to the desired pressure. Subsequently, the compressed CO₂ was heated in a separate storage tank to the desired temperature and thus brought into supercritical conditions. The autoclaves were pressurized simultaneously by thus conditioned CO₂ using a pressurization rate of approx. 1.5 MPa/min via the control valve 15 (Figure 2). Afterwards, the inlet valve of each autoclave was closed to conduct separated experiments. The starting time for the adsorption process was taken when constant temperature and pressure were reached. Pressures between 10 and 18 MPa and temperatures in the range of 35 to 60 °C were investigated. At the end of the adsorption process, the autoclaves were simultaneously depressurized via the heated control valve 16 (Figure 2) using a depressurization rate equal or smaller than -0.1 MPa/min to avoid precipitation of the solubilized fish oil in the depressurized CO₂. The fish oil loaded aerogel particles were stored still packed in filter paper in a desiccator for further analysis.

The second set of static adsorption experiments was needed to load enough aerogel particles for consecutive fish oil oxidation measurements. It was conducted at the system conditions, where the highest fish oil loading could be achieved during the first set of experiments. A 1 L high pressure autoclave was used to load 12 g of aerogel particles via adsorption of fish oil from supercritical CO₂. Therefore, 16 g fish oil were placed with a syringe at the bottom of the preheated autoclave (40 °C). Afterwards, a metal lattice was placed above the fish oil. 4 g of each aerogel particle source (WPI, EWP and NaCas) were put into filter papers and placed onto the metal lattice without any contact to the fish oil. The autoclave was closed and pressurized by CO₂ up to 18 MPa. After 72 h the depressurization (- dp/dt < 0.1 MPa/min) was started. The loading experiment in the 1 L autoclave was carried out twice.

2.2.3. Analysis of fish oil loading

The complete sample amount (adsorption in 40 mL autoclaves) or 0.6 g (adsorption in 1 L autoclave) of the loaded aerogel particles were placed in 30 mL hexane in a 50 mL centrifuge tube, sonicated for 15 min and centrifuged (800 rcf) for 15 min. 20 mL of the fish

oil hexane mixture was transferred to a round-bottomed flask (volume = 250 mL). 20 mL fresh hexane was added to the centrifuge tube, sonicated and centrifuged. Again 20 mL of the fish oil hexane mixture was transferred to the same round-bottomed flask. This extraction step was repeated four times resulting in 80 mL fish oil hexane mixture in the round-bottomed flask. The hexane was evaporated afterwards in a rotating evaporator at 55°C and 0.02 MPa. The fish oil amount in the round-bottomed flask was determined gravimetrically. Duplicate measurements were conducted for the samples being processed in the 1 L autoclave.

2.2.4. Analysis of fish oil

Lipid composition of the fish oil was determined as follows: 50 to 100 mg fish oil was weighed in a 1 mL-volumetric flask (± 1 mg) and dissolved in chloroform. 150 μ L of this solution and 150 μ L of N-methyl-N-trimethylsilylheptafluorobutyramide (MSHFBA) were transferred to a GC vial where active hydrogen groups were derivatized to form volatile trimethylsilyl-derivatives. Therefore, the sample was placed for one hour at 60 °C in an oven before being analyzed by gas chromatography. The utilized GC-FID method is described in Table 2.

Table 2: Settings GC/FID system for fish oil fat analysis.

Instrument:	Agilent GC7890B with FID, autosampler 7683		
Column:	DB 17HT, 30 m x 0.15 mm I.D. x 0.32 μ m from Agilent Technologies, Inc. (Santa Clara, USA)		
Carrier gas:	Helium (99.999% purity)		
Temperature program:	Temperature (°C)	Time (min.)	Rate (°C/min)
	50	1	13
	240	0	15
	310	0	40
	348	38	-
Gas setting:	carrier gas: He=0.08 MPa; detector: combustion gas H ₂ =40 mL/min, oxidant gas air=450 mL/min, makeup gas He=45 mL/min		
Injection conditions:	350 °C, split ratio 1:10		
Injection volume:	1 μ L		
Detector temperature:	350°C		

The resulting chromatogram was evaluated by comparison with standard chromatograms. Therefore, stock solutions of known lipid components (approx. 2 g/L free fatty acids (FFA) and monoglycerides, 2-4 g/L diglycerides and 4-6 g/L triglycerides) or cholesterol were dissolved in chloroform and subsequently derivatized and analyzed as described above. The results are reported as cumulated peak areas of the corresponding lipid component relative to the total cumulated peak areas.

The determination of the fish oil fatty acid composition was conducted according to DIN EN ISO 12966-2 (DIN-Normenausschuss Lebensmittel und landwirtschaftliche Produkte, 2017) via transesterification of the fish oil into the corresponding fatty acid methyl esters (FAME) and subsequent gas chromatographic (GC) analysis (Simon and Stahl, 2016).

2.2.5. Oxidation measurements of fish oil loaded particles

The volatile compounds of the fish oil loaded aerogel particles were analyzed using static headspace gas chromatography coupled to flame ionization detection (SHS-GC-FID). The analysis was conducted directly after the adsorption experiments. First, the fish oil loaded aerogel particles were transferred into 20 mL vials which were sealed hermetically. The vials were heated up to 100 °C for one hour in the headspace system (TurboMatrix HS40 Trap, PerkinElmer, Inc., Waltham, USA) to equilibrate the volatile compounds of the sample and the corresponding headspace. Afterwards, a dosing needle was inserted into the vial and the pressure was raised to 0.1379 MPa for one minute to transfer the headspace to the GC-FID system. The settings of the utilized GC method are presented in Table 3. The measurements were repeated twice for each sample.

Table 3: Settings GC/FID system for the determination of the volatile compounds.

Instrument:			
Agilent GC7890A with FID			
Column:	Stabilwax-DA, 60 m x 0.32 mm I.D. x 1.00 µm from Restek GmbH (Bad Homburg v. d. Höhe, Germany)		
Carrier gas:	Helium (99.999% purity)		
Temperature program:	Temperature (°C)	Time (min.)	Rate (°C/min)
	40	1	10
	210	12	-
Gas setting:	carrier gas: He=0.08 MPa; detector: combustion gas H ₂ =40 mL/min, oxidant gas air=400 mL/min, makeup gas He=45 mL/min		
Injection temperature:	150°C		
Detector temperature:	250°C		

The resulting chromatograms were compared with standard substances to identify the detected compounds using the measured retention times. Hereby the focus was on the substances identified by LEE ET AL. as odor intense oxidation products of ω-3-fatty acids (Lee et al., 2003).

To get more detailed information about the composition of the volatile compounds and the oxidative status of the fish oil, loaded protein aerogel particles were evaluated for selected samples by head space solid-phase microextraction followed by a gas chromatography-mass spectrometry (HS-SPME-GC-MS) analysis. The used SPME-GC-MS method and its

performance control is described in detail in LEHTONEN ET AL. (LEHTONEN ET AL., 2016). Triplicate measurements were done using 0.5 g of the dry, fish oil loaded aerogel particles per measurement. The fish oil and fish oil loaded samples were sent in dry atmosphere from Hamburg to Helsinki within 3-4 days. They were analyzed approx. one week after arrival and stored in the fridge (+4 °C) prior to analysis. The results are reported as average peak areas over three replicate samples.

2.2.6. Storage at defined relative humidity

Storage experiments were conducted at a relative humidity (rH) of 11% and 33% and at room temperature (20 °C), representing dry storage conditions. WPI, EWP and NaCas aerogel particles without loading and fish oil loaded WPI and EWP particles were stored over 2, 4, 8 and 12 weeks. The respective rH was created with saturated salt solutions of LiCl (rH 11%) and MgCl₂ (rH 33%) (Greenspan, 1977). Small, flat bottomed dishes were filled with a layer of about 2 g of particles and placed on a rack just above the surface of the salt solution in sealable containers. An absorption or desorption of water was determined gravimetrically.

3. Results and discussion

3.1. Loading of aerogel particles by adsorption of fish oil from supercritical CO₂

First, the kinetics of the static fish oil adsorption from supercritical CO₂ were measured at the highest pressure (18 MPa) and lowest investigated temperature (40 °C) to determine the longest equilibration time in the utilized high-pressure equipment since adsorption is generally favored at these conditions. When investigating other system conditions, it was assumed that the equilibrium was reached in any case when the time was longer than the equilibration time at 18 MPa and 40 °C. The adsorption kinetic results for the investigated carrier materials are presented in form of the fish oil loading (loaded fish oil mass per mass of unloaded/pure aerogel micro-particles) in Figure 3.

The highest fish oil loadings (up to 0.74 g fish oil/g aerogel) were achieved for EWP-aerogels and WPI I-aerogels. The corresponding thermodynamic equilibria were reached after 48 h. Lower fish oil loadings per gram aerogel and shorter equilibration times (16 h and 20 h) were observed for the NaCas-aerogels and the WPI II-aerogels.

In principal three loading mechanisms of the porous aerogel structure are conceivable: adsorption, capillary condensation (including adsorption) or absorption (entrapment of fish oil molecules in solid protein matrix). In case of loading the protein aerogels with fish oil, it is not possible to exclude any mechanism or a combination of mechanisms. In our opinion, the adsorption of fish oil molecules onto the high inner surface area of the aerogel in combination

with a partially capillary condensation seems most reasonable. In case of complete capillary condensation, we would have expected higher fish oil loadings: using a fish oil density of 925 kg fish oil/m³ and the pore volume of WPI I-aerogel particles (0.00155 m³/kg aerogel) would lead for example to a loading of 1.43 g fish oil/g aerogel. The absorption of fish oil in the solid protein aerogel backbone seem rather improbable. Although we cannot exclude any of the loading mechanism, we used the word “adsorption” in this publication as synonym for the most probable aerogel loading mechanism (adsorption combined with partially capillary condensation).

In a second step, the influence of pressure and temperature was investigated. The temperature range was limited to 31 °C (critical temperature of CO₂) and 60 °C (lowest denaturing temperature of the utilized proteins). Within this range only a slight influence of the temperature on the equilibrium fish oil loading could be observed (increasing temperature led to decreasing loading; data not shown). The pressure influence on the equilibrium fish oil loadings for different aerogel matrices is shown in Figure 4. As expected, the equilibrium fish oil loading increases for all carrier matrices with increasing system pressure similar to the findings of COMIN ET AL., who investigated the static supercritical CO₂ impregnation of barley β -glucan aerogels with flax oil (Comin et al., 2012). A higher system pressure shifts the equilibrium of the fish oil being solubilized in the supercritical CO₂ bulk phase or being adsorbed on the aerogel surface towards the adsorbed fish oil on the aerogel surface. Additionally, the solubility of fish oil in supercritical CO₂ increases with increasing system pressure (Gupta and Shim, 2006) allowing higher loadings on the aerogel surface. COMIN ET AL. determined an oil loading of approx. 0.887 g flax oil/g aerogel at 15 MPa and 40 °C for the static impregnation process (Comin et al., 2012) which is higher than the fish oil loadings measured in this study (Figure 4). One explanation could be that COMIN ET AL. used fast depressurization rates in their study, resulting into oil precipitation and thus high aerogel loadings. The precipitation phenomenon is well known when the pressure is released relatively fast for compounds which are soluble in dense CO₂ (Gurikov and Smirnova, 2018). Another reason could be that the aerogel structure of barley β -glucan aerogels favors the oil loading more than the protein aerogel structures. Since the barley β -glucan aerogel structure was not characterized in detail (Comin et al., 2012) no structure comparison is possible here. We tried to avoid oil precipitation using slow depressurization rates ($- dp/dt < 0.1$ MPa/min) to avoid agglomerated particles due to precipitated (fish) oil onto the particles outer surface to retain the free-flowing properties of the aerogel particles. Faster depressurization rates were tried which resulted in higher fish oil loadings, precipitated fish oil onto the particles outer surface and agglomerated particles. The particles seemed “wet” and did not retain their free-flowing properties.

Since the adsorption is dependent on the accessible inner surface of the aerogel particles, the loaded fish oil mass was related to the respective BET-surface area (Figure 5). Additionally, the pressure related fish oil solubility in supercritical CO₂ (Gupta and Shim, 2006) was used instead of the system pressure to construct isotherms using the Freundlich and Langmuir approach (Foo and Hameed, 2010) (Figure 5). This isotherm construction differs from the studies of PANTIĆ ET AL. and MARCO AND REVERCHON, where a constant pressure and concentrations below the saturation concentration in supercritical CO₂ were used to apply Langmuir and Freundlich models for the impregnation of alginate and starch aerogels with vitamins (Marco and Reverchon, 2017; Pantić et al., 2016a). Thus, a meaningful comparison to their findings does not seem reasonable. As expected, the specific loadings increase with increasing fish oil solubility in CO₂ (Figure 5). Additionally, different WPI-aerogels (WPI I and II) having different BET-surface areas show similar loadings per BET-surface area. Therefore, it can be concluded that an increase of the BET-surface area leads generally to higher loadings per gram aerogel. The specific loadings on the EWP- and NaCas-aerogels were described more precisely by the Freundlich model than by the Langmuir model whereas the specific loading on the WPI-aerogels could not be described adequately by both investigated models.

A possible, simple alternative to the adsorption from supercritical CO₂ is to load the aerogel particles by direct mixing with fish oil (details are given in the Supplementary information). This method led to significantly higher loadings, but large parts of the fish oil stick to the outside of the particles as liquid bridges between the aerogel particles instead of being sorbed by the porous matrices (Figure 6). Thus, the flowability of the powder is significantly decreased and no protection of the fish oil from oxidation can be expected. In comparison, the supercritical CO₂ loading method avoids liquid bridges, since the fish oil is rather adsorbed on the inner and outer surface of the aerogel matrices and the particles stay dry and free-flowing (as proven for all three loaded protein sources, Figure 6). We believe, these phenomena are advantageous in later industrial applications, where the powder flowability plays a role.

AHMADI ET AL. presented another fish oil loading method (Ahmadi et al., 2016): Whey protein gel monoliths were freeze dried to cryogels (BET-surface areas < 5 m²/g) and soaked in a hexane-fish oil-solution whereby the hexane was evaporated subsequently. The achieved specific loading of AHMADI ET AL. (0.013 g fish oil/m² cryogel) is seven times higher than the fish oil adsorption from supercritical CO₂ at 40 °C and 18 MPa onto WPI-aerogel particles, but the absolute loading (0.026 g fish oil/g cryogel) is more than one order smaller (Ahmadi et al., 2016).

Overall, loadings up to 0.74 g fish oil/g aerogel could be achieved onto the protein aerogels using static supercritical CO₂ impregnation. Slightly higher loadings (0.916 g oil/g aerogel) were reached for the loading of flax oil onto barley β -glucan aerogels (Comin et al., 2012) showing that protein aerogels exhibit a similar potential as carrier material for oils compared to polysaccharide aerogels. Compared to the vitamin loadings of polysaccharide-based aerogels the fish oil loadings on protein aerogels show more than three times higher values (Haimer et al., 2010; Marco and Reverchon, 2017; Pantić et al., 2016b; Pantić et al., 2016a). Additionally, the achieved fish oil loading is higher than the usual loading of 0.333 to 0.5 g fish oil/g wall material reported for the microencapsulation of fish oil by spray drying (Encina et al., 2016). For a later industrial application, the diffusion driven static adsorption from supercritical CO₂ should definitely be replaced by a dynamic adsorption to shorten the process time. A circulation pump could accelerate the mass transfer of valuable compounds in the fluid phase by convection.

3.2. Fat composition of loaded fish oil

Since fish oil is a mixture of different tri-, di-, monoglycerides and free fatty acids the question arises, whether the dissolution in supercritical CO₂ or the adsorption are selective or non-selective. Therefore, the compositions of the fresh fish oil, the fish oil from the aerogel particles and from the autoclave were analyzed. Figure 7 shows that tri-glycerides were adsorbed in a higher proportion and free fatty acids in a lower proportion compared to the composition of the fresh fish oil. Additionally, the cholesterol proportion is slightly higher for all three loaded aerogel matrices compared to the fresh fish oil and corresponding fish oil residues in the autoclave. Further analyses of the fish oil compositions were conducted for all three protein matrices at different adsorption pressures and adsorption times, but no clear trends could be observed hereby. This is positive for the process development, because it is possible to choose the process conditions without effects on the fish oil composition.

The fatty acid composition of the utilized fish oil and fish oil loaded WPI- and EWP-aerogel particles was analyzed, whereas the absolute mass of fish oil being adsorbed on the surface of the NaCas-aerogel particles was too low for analysis. Figure 8 shows that the utilized fish oil exhibits a similar fatty acid profile as the adsorbed fish oil on the aerogel particles' surfaces and the fish oil residues in the autoclave (non-adsorbed fish oil). Thus, the fatty acid distribution changes only slightly during the adsorption process of fish oil from supercritical CO₂. A slightly smaller proportion of the essential ω -3-fatty acids (C20:5 and C22:6) was determined in case of the loaded EWP-aerogels. One reason for this could be that a small portion of the sensitive ω -3-fatty acids was oxidized after the adsorption onto the EWP-surface and thus was decomposed into volatile oxidation products (Section 3.3). For the WPI-aerogel particles the fish oil adsorption is non-selective. Its high ω -3-fatty acid content is

similar to the high content of C20:5 and C22:6 in micro encapsulated tuna oil (spray dried tuna oil-in-water-emulsions) (Shen et al., 2010). Thus, the requirement to load high portions of ω -3-fatty acids is fulfilled for the supercritical CO₂ impregnation of aerogels as new loading technique for fish oil.

3.3. Volatile compounds of loaded fish oil

To investigate the odor and the oxidative status of the fish oil loaded aerogel particles the volatile compounds were examined by static head-space GC-FID. Propanal, pentanal and 1-penten-3-ol were identified in the head spaces of the utilized fish oil and fish oil loaded aerogels. These compounds as well as 2-pentenal being similar to pentanal are well-known as volatile oxidation products of ω -3-fatty acids of fish oil (Miyashita et al., 2018). The highest volatiles per gram fish oil were measured for the fish oil (Figure 9), indicating that the odor of the pure fish oil is higher than for the fish oil being adsorbed on the surface of the aerogel matrices. A reason for that could be that the fish oil is stabilized by the adsorption and thus less oxidized when heated up to 100 °C for one hour (during head-space GC analysis). Another reason could be that the smaller volatile molecules were flushed out during the depressurization of the supercritical CO₂ or were adsorbed in a lower proportion than the larger non-volatile molecules (cf. Figure 7). Comparing the loaded aerogels, the WPI-aerogel particles showed the lowest and NaCas-aerogel particles the highest quantity of volatiles per gram loaded fish oil (Figure 9).

To analyze the fish oil volatiles at lower temperature, HS-SPME-GC-MS measurements were conducted. High temperatures favor the oxidation of the ω -3 fatty acids and lead to degradation of the oxidation products (Miyashita et al., 2018). The used SPME procedure releases and extracts the volatiles at low temperature (40 °C) within 40 min (Lehtonen et al., 2016), but it is selective since low boiling compounds may be discriminated during the injection into the GC and thus favors the extraction of higher boiling compounds (up to 250 °C) (Lehtonen et al., 2016; Miyashita et al., 2018). Therefore, the previously determined volatiles (propanal, pentanal and 1-penten-3-ol) could not or only to a small extent be detected (details are given in the supplementary information). Overall, remarkable high amounts of ethanol (and its denaturant 2-butanone (MEK) (Kleemann et al., 2018)) could be observed for all unloaded aerogel particles. The drying of the aerogel particles seems not to remove the denatured ethanol completely. It might stay adsorbed on the high specific surface area of the aerogels. Nevertheless, the aerogel properties lay in the expected range and longer dryings did not change the properties significantly. In later industrial applications ethanol without any denaturant can be used, so that the MEK as denaturing substance is of minor importance for industrial food applications.

To get an impression of the volatiles contained in the fish oil, the unloaded and fish oil loaded protein aerogel particles, the summarized identified volatiles (without ethanol), the volatiles classified as oxidative markers (Gómez-Cortés et al., 2015; Miyashita et al., 2018) and the volatile ethanol in the respective samples (detailed results in supplementary information) are shown in Figure 10 and Figure 11.

As expected, the pure, cooled fish oil showed the lowest amounts of oxidative markers and ethanol while the oxidative status of the (non-adsorbed) fish oil remaining in the autoclave increases during the adsorption process (Figure 10) probably due to the raised temperature in the autoclave (40 °C compared to 3 °C in the fridge). The total volatile content of the fish oil is in the same range as for unloaded WPI- and NaCas-aerogel particles (Figure 10). The high content of oxidative markers in case of all unloaded aerogel particles could be explained by a high contact area between protein gel particles and sun flower oil during the water (protein solution) in oil emulsion gelation step within production (Kleemann et al., 2018). Although the gel particles were separated from most of the oil after the gelation it seems that the oil residues stick to the particles' outer surfaces, get in contact with oxygen after drying and thus smell.

The ethanol smell of the aerogels is reduced during the adsorption process of fish oil from supercritical CO₂ (Figure 10) since ethanol is miscible with scCO₂ at the utilized adsorption conditions. Thus, the ethanol content between the different aerogel particles (being processed in the same autoclave) and the remaining fish oil at the bottom of the autoclave is equilibrated. This effect seems to be independent of the aerogel's pore structure and loaded fish oil mass (Figure 11). For the total amount of volatiles (without ethanol) no clear trend is observable (Figure 10): It increases for WPI- and NaCas-aerogel particles and decreases for EWP-aerogel particles during the adsorption process. However, the total amount of oxidative markers decreases for all investigated aerogel particles during the loading (Figure 10). It seems that the supercritical CO₂ extracts large portions of the initial volatiles ($\triangleq$ volatiles of unloaded aerogels) from the aerogels since a higher pressure (18 MPa) and thus a higher CO₂ density and solvating power is used during the fish oil impregnation process compared to the supercritical drying process (12 MPa), which is used beforehand to produce the aerogels. Additionally, the initial volatiles in the porous aerogel structure could be flushed out during pressurization and depressurization of the impregnation process.

Due to varying fish oil loadings the volatiles were related to the containing fish oil mass and compared with the fish oil before and after the adsorption process (Figure 11): WPI- and EWP-aerogels show a similar oxidative status like the autoclaved fish oil, whereas NaCas-aerogels exhibit the highest content of total volatiles, oxidative markers and ethanol. One reason for this could be a difference in hydrophobicity of the aerogels' inner surfaces. In case

of higher hydrophobicity, stronger interactions with the fish oil molecules and thus a lower content of volatiles and oxidation are expected. During heat induced gelation, hydrophobic sites of the WPI- and EWP-proteins become exposed to the protein surface and interact intra- or intermolecularly, whereas enzymatic crosslinking of NaCas does not increase the hydrophobicity of the proteins surface (Kleemann et al., 2018). For this reason and their low loading capacity (Section 3.1) it is recommended to use WPI- or EWP-aerogel particles as carrier material instead of NaCas-aerogel particles.

In summary, WPI- and EWP-aerogels are possible carrier materials for fish oil regarding loading capacity and oxidative status of the loaded fish oil. Nevertheless, the oxidative status of the fish oil loaded on the particles is higher than the untreated fish oil stored at 3 °C. A loading of the fish oil onto aerogel particles may reduce oxidation in applications, where the fish oil containing food is heated up (cooked, roasted, grilled or baked). To prevent fish oil oxidation the loaded aerogel particles could be coated subsequently (Goslinska et al., 2019) to encapsulate the fish oil within the aerogel particles as shown by AHMADI ET AL. for WPI-cryogels (Ahmadi et al., 2016).

3.4. Storage behavior of fish oil loaded protein aerogel particles

The shelf life of the unloaded protein aerogel particles is relevant, when the particles are produced in advance and the loading is conducted elsewhere. The storage of unloaded WPI-, EWP- and NaCas-aerogel particles was conducted at a relative humidity (rH) of 11 % and 33 % for 12 weeks. The weight gain due to water uptake was less than 1 % and no distinct optical changes were observed for all three protein systems during storage time. WPI and NaCas particles in particular kept their very good free flowing properties, remained unchanged and only little dust was observed. EWP particles showed a slightly cemented layer on the top of long stored samples. However, these clumps easily dissociated when agitated. EWP particles generated a lot more dust because of their small particle size.

Storage experiments of fish oil loaded aerogel particles reveal the protective properties of the aerogel particles and possible changings during storage. WPI- and EWP-aerogel particles, which were loaded with fish oil from supercritical CO₂, showed similar storage behavior as the unloaded particles. The relative weight change was for all tested samples about 1% (see supplementary information). No fish oil leaked from the particles during the entire storage time and the loaded particles kept their free-flowing properties.

The variation of volatile compounds of the fish oil loaded WPI- and EWP-aerogel particles was determined by means of SHS-GC-FID analysis. A slight increase of the sum of identified volatiles (without ethanol) can be observed for both aerogel systems during storage (Figure 12), whereby the higher relative humidity and the adsorption on EWP-aerogel particles

enhances the quantity of volatiles. The sum of volatiles of the fish oil utilized for the loading is two orders of magnitude higher (cf. Figure 9) than for the fish oil loaded aerogel particles being stored (Figure 12). Since the samples were heated up during the analysis it can be concluded that the stabilizing effect of the adsorption for the fish oil at raised temperatures maintains over the storage time of 12 weeks.

Overall, the fish oil is bound to the network structure of the protein aerogel particles and the microcapsules represent a stable product over a storage time of 12 weeks.

4. Conclusions and outlook

The potential of protein aerogels as carrier material for food additives was investigated. Protein aerogel micro-particles were successfully loaded with fish oil by static supercritical CO₂ impregnation. Loadings up to 0.74 g fish oil/g aerogel could be achieved for WPI- and EWP-aerogels while the aerogel micro-particles retained their free-flowing properties and the fish oil retained its composition. Additionally, the loaded fish oil showed less volatile oxidation products of ω -3 fatty acids compared to free/unbound fish oil at high temperature. Fish oil loaded WPI-aerogels exhibited slightly higher proportions ω -3 fatty acids and slightly lower volatile oxidation products of ω -3 fatty acids compared to fish oil loaded EWP-aerogels. NaCas-aerogels showed the lowest fish oil loading and the highest ratio of volatiles related to the loaded fish oil mass. During a storage time of 12 weeks the fish oil loaded aerogel particles kept their free-flowing properties representing a stable product without any fish oil leaking.

Overall the protein aerogel micro-particles are suitable as carrier material for sensitive oils. To reduce the oxidation of the loaded (fish) oil further a second compound could be co-impregnated as antioxidant (for example tocopherols (Miyashita et al., 2018)) or an additional coating could be applied in future studies. Additionally, a dynamic process instead of a static supercritical CO₂ impregnation process should be investigated to decrease the process time.

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Notes

Declarations of interest: none

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Figure 1: Scanning electron micrographs of utilized WPI-, EWP-, NaCas-aerogel particles (5 kV, magnification: 200 and 2000).

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Figure 3: Adsorption kinetics of the fish oil from supercritical CO₂ onto WPI-, EWP- and NaCas-aerogel particles. Adsorption conditions: P=18 MPa, T=313 K. The lines are used to guide the eye (WPI I: black solid line, WPI II: black dashed line, EWP: grey dashed line, NaCas: black dotted line).

Figure 4: Fish oil loading as function of the system pressure onto WPI-, EWP- and NaCas-aerogel particles. Adsorption conditions: $T=313\text{ K}$, $t \geq t_{\text{equilibrium}}$

Figure 5: Specific fish oil loading onto WPI-, EWP- and NaCas-aerogel particles related to the corresponding BET-surface area of the aerogel as function of the fish oil solubility in CO_2 in comparison with the corresponding Freundlich isotherms (solid lines) and Langmuir isotherms (dashed lines). Adsorption conditions: $T=313\text{ K}$, $t \geq t_{\text{equilibrium}}$, $10\text{ MPa} < P \leq 18.5\text{ MPa}$.

	Aerogel particles without fish oil	Aerogel particles with fish oil	
		Adsorption from supercritical CO ₂ [g _{fish oil} /g _{aerogel}]	Mixing with fish oil [g _{fish oil} /g _{aerogel}]
WPI I		0.618 ± 0.028	3.04 ± 0.14
WPI II		0.276 ± 0.031	1.13 ± 0.03
EWP		0.739 ± 0.022	4.91 ± 0.12
NaCas		0.169 ± 0.001	0.79 ± 0.01

Figure 6: Comparison of photographs taken with a light microscope of unloaded and fish oil loaded WPI-, EWP- and NaCas-aerogel particles (adsorption from supercritical CO₂ and direct mixing with fish oil).

Figure 7: Composition of the fresh fish oil, the adsorbed and non-adsorbed fish oil for WPI-, EWP- and NaCas-aerogels. Adsorption conditions: $P=18$ MPa, $T=313$ K, $t = 48$ h.

Figure 8: Fatty acid methyl ester distribution of the fresh fish oil, the adsorbed and non-adsorbed fish oil for WPI- and EWP-aerogels. Adsorption conditions: $P=18$ MPa, $T=313$ K, $t \geq t_{\text{equilibrium}}$. Ω -3-fatty acids are marked in red.

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Encapsulation of fish oil in protein aerogel micro-particles

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Highlights

- Protein aerogel micro-particles were impregnated from supercritical CO₂ solution
- Specific loadings up to 0.74 g fish oil/g aerogel were achieved
- Fish oil loaded aerogel micro-particles showed free-flowing properties
- Fish oil composition and FAMEs could be maintained