

Hydrophobic Solubilization in Aqueous Colloidal Systems

Mechanistic Insights into Protein-Based Solubilization and

Additive-Modified Surfactant-free Microemulsions



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Abstract

The solubilization of hydrophobic compounds in water is a common challenge in industry and daily life. In this work, different solubilization strategies are examined, and experimental findings are linked to schematic mechanistic concepts. This aims to contribute to more sustainable solubilization methods. In the first chapter, the topic is introduced, and a comparative overview of the most important techniques is provided. In the second chapter, the solubilization of commercially available, denatured plant protein isolates in aqueous media is investigated. Three isolates from pea, chickpea, and potato are subjected to various physical and chemical modification methods, as well as combinations of these, to identify the solubility-limiting factors hydrophobicity and aggregation. The protein solubility of all tested isolates is significantly improved by targeting both non-covalent and covalent bonds: combining SDS with pH shifting destabilizes insoluble aggregates through protein unfolding and enhances interaction with SDS. A schematic solubilization mechanism is proposed, which may also apply to other plant protein isolate classes. In the third chapter, pea protein isolate is used to solubilize the polyphenol curcumin in water. Using a well-established pH-shifting method, supersaturated curcumin-loaded pea protein isolate nanoparticles are prepared. While the curcumin concentration immediately after preparation is 1.39 ± 0.093 mg/mL, it decreases to only 0.0528 ± 0.0088 mg/mL after 24 hours of continuous stirring. Fundamental studies into supersaturation reveal an underestimated influence of the nanoparticle preparation method on curcumin binding. Precipitation of the polyphenol from the largely inert protein carrier is attributed to either aggregation of tightly packed curcumin molecules on the carrier surface or their isomerization followed by aggregation. Based on these findings, the prepared nanoparticles are proposed as a supersaturated drug-delivery system (SDDS). A schematic solubilization mechanism is proposed, which may also apply to the solubilization of curcumin with other plant protein isolate classes. Emphasizing the formulation's kinetic and stable states allows for diverse applications in both food and pharmaceutical industries. In the fourth chapter, the effect of low-concentration surface-active additives on the binodal and the mesostructure of the surfactant-free microemulsion EtOH/1-Octanol/Water is investigated. While the addition of nonionic surfactants does not notably affect the binodal, the addition of ionic surfactants and an ionic hydrotrope causes a significant decrease. Building on previous research, the impact of an additive on the binodal is proposed to depend on whether it carries a charge, its solubility in the individual solvent compounds, and, for ionic surfactants, the headgroup's hydration. Changes in the mesostructure are assessed by evaluating SAXS data. This approach maintains the system's solubilization capacity for a hydrophobic compound while increasing water content and reducing the amount of organic solvent by adding small amounts of ionic compounds.

Zusammenfassung

Die Solubilisierung hydrophober Komponenten in Wasser ist eine zentrale Herausforderung in der Industrie und im Alltag. In dieser Arbeit werden verschiedene Solubilisierungsstrategien untersucht und experimentelle Ergebnisse mit mechanistischen Konzepten verknüpft, um einen Beitrag zur nachhaltigeren Solubilisierung zu leisten. Im ersten Kapitel wird die Thematik eingeführt und ein Überblick über die wichtigsten Methoden gegeben. Im zweiten Kapitel wird die Solubilisierung kommerziell erhältlicher, denaturierter Pflanzenproteinisolate in wässrigen Systemen untersucht. Erbsen-, Kichererbsen- und Kartoffelproteinisolate werden physikalischen und chemischen Modifikationen sowie Kombinationen daraus unterzogen, um Hydrophobie und Aggregation als Löslichkeitslimitierende Faktoren zu identifizieren. Die Proteinlöslichkeit aller untersuchten Isolate kann durch das kombinierte Ansprechen kovalenter und nichtkovalenter Bindungen signifikant erhöht werden: Die Kombination aus SDS und einer pH-Verschiebung destabilisiert unlösliche Aggregate durch Proteinentfaltung und verbesserte Interaktion mit SDS. Ein schematischer Mechanismus wird vorgeschlagen, der auf andere Pflanzenproteinisolate übertragbar sein könnte. Im dritten Kapitel wird Erbsenproteinisolat verwendet, um Curcumin in Wasser zu lösen. Mit Curcumin beladene Erbsenproteinisolatnanopartikel werden mittels einer etablierten pH-Verschiebungsmethode hergestellt. Innerhalb von 24 Stunden unter konstantem Rühren sinkt die Curcuminkonzentration in Wasser von $1,39 \pm 0,093$ mg/mL auf $0,0528 \pm 0,0088$ mg/mL. Fundamentale Untersuchungen zur Übersättigung zeigen einen unterschätzten Einfluss der Herstellungsmethode auf die Anbindung von Curcumin. Das Ausfallen wurde auf die Aggregation dicht gepackter Curcuminmoleküle an der Nanopartikeloberfläche oder auf deren Isomerisierung und die nachfolgende Aggregation zurückgeführt – eine Beteiligung des Proteinträgers kann größtenteils ausgeschlossen werden. Das Herausarbeiten der kinetischen und stabilen Zustände der Formulierung ermöglicht einen breiten Einsatz in der Lebensmittel- und Pharmaindustrie. Im vierten Kapitel wird der Effekt geringer Konzentrationen grenzflächenaktiver Komponenten auf die flüssig-flüssig-Phasengrenze und die Mesostruktur der tensidfreien Mikroemulsion EtOH/1-Octanol/Wasser untersucht. Während die Zugabe von nichtionischen Tensiden keinen nennenswerten Effekt auf die Phasengrenze hat, senkt die Zugabe von ionischen Tensiden und einem ionischen Hydrotrop die Phasengrenze signifikant. Aufbauend auf vorherigen Arbeiten wird vorgeschlagen, dass der Effekt eines Additivs auf die Phasengrenze anhand des Tragens einer Ladung, der Löslichkeit in den einzelnen Lösungsmittelkomponenten und, im Falle ionischer Tenside, der Hydratisierung der Kopfgruppe abgeschätzt werden kann. Unterschiede in der Mesostruktur wurden anhand von SAXS-Daten herausgearbeitet. Dieser Ansatz bewahrt die Solubilisierungsfähigkeit des Systems, erhöht gleichzeitig den Wassergehalt und reduziert die Menge an organischem Lösungsmittel.

List of Abbreviations

β_g	Gelation concentration
t0	Directly after preparation
t24	after 24 hours
T _d	Denaturation temperature
T _g	Gelling temperature
Tvalue→22	Heating at a certain temperature (value) and shifting back to 22 °C
μE	microemulsion

ADME	Adsorption, distribution, metabolism, and excretion
BDMC	Bisdemethoxycurcumin
BE	Binding efficiency
BP	Broad peak
C12E10	Polyoxyl-10 lauryl ether
CD	Circular dichroism
CMC	Critical micellar concentration
CPIp	Chickpea protein isolate
CTAB	Cetyltrimethylammonium bromide
Cur	Curcumin
DLS	Dynamic Light scattering
DLVO	Derjaguin–Landau–Verwey–Overbeek
DSC	Differential scanning calorimetry
ESRF	European Synchrotron Radiation Facility
EtOH	Ethanol
FAO	Food and Agriculture Organization of the United Nations
GML	Glycerol monolaurin
GRAS	Generally recognized as safe
IMP	improbable or invalid metabolic panacea
IR	Infrared spectroscopy
LA	loading amount
LTAB	Lauryltrimethylammonium bromide

MCT	Medium chain triglyceride
MD	molecular dynamics
Meg	N-methyl-D-glucamine, Meglumine
MHC	minimal hydrotropic concentration
MS	Mass spectrometry
NaL	Sodium laurate
NaSal	Sodium salicylate
NTA	Nanoparticle Tracking Analysis
NSI	nitrogen solubility index
Nstir	not stirred
Oct	1-Octanol
o/w	Oil in water
OZ	Ornstein-Zernike
PAINS	pan-assay interference compound
PDI	Polydispersity index
pHvalue→n	pH shifting from a certain pH (value) to the native pH
pI	isoelectric point
PFJ	potato fruit juice
PPI	Pea protein isolate nanoparticles
PPIp	Pea protein isolate
PPICur	pea protein isolate-curcumin nanoparticles
PoPIp	Potato protein isolate
RT	room temperature
red	redispersed
SANS	small-angle neutron scattering
S(W)AXS	small-(and-wide)-angle X-ray scattering
SDDS	supersaturated drug delivery system
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
SEC	Size-exclusion chromatography
SFME	Surfactant-free microemulsion
SIF	Simulated intestinal fluid
SLEC	Sodium laureth-6 carboxylate
VIII	

SLS	Static light scattering
SPI	soy protein isolate
stir	stirred
TGA	Thermogravimetric analysis
TS	Teubner-Strey
US	Sonication
VitC	Vitamin C, L (+)-ascorbic acid
(v/v)	volume percent
w/o	Water in oil
WPI	whey protein isolate
(w/w)	weight percent
XRD	X-Ray Diffraction

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Chapter 1 General Background

1.1. Introduction

The immiscibility of two liquids, such as oil and water, is a common phenomenon. Instead of forming a uniform solution through mixing, two separate phases appear. Solubilizing oil in water is of everyday interest, for example, in cleaning or cooking, but it is also a major concern in various industrial sectors. In the pharmaceutical industry, it is estimated that 70-90% of new drug candidates are insoluble or poorly soluble in water¹. In the transition from medicine to nutrition, dietary supplements and functional foods, i.e. isolated substances with health benefits or substances added to food to enhance nutritional value², respectively, have gained significant attention in recent years. Polyphenols are popular candidates for formulation due to their well-known antioxidant properties. However, their water-solubility is low, and whether dealing with polyphenols or drugs, the food and pharmaceutical industries must address this challenge to ensure absorption¹. The problem of aqueous solubility also extends to the chemical industry, for example, in the case of poorly water-soluble monomers for polymerization in plastic manufacturing, or product formulation in the cosmetic industry.

The cause of these issues can be explained by differences in molecular structure and the resulting molecular interactions in water and oil, for example. Water is a polar molecule and interacts strongly with other water molecules through hydrogen bonds. Oil compounds are nonpolar and interact with each other via van der Waals forces. Only molecules that share the same type of interactions can form molecular solutions because their interactions are favorable. This basic principle is summarized by the phrase “like dissolves like”.

To solubilize molecules with opposite polarities, this interaction barrier must be overcome. This can be achieved by adding amphiphilic molecules that exhibit both polar and nonpolar parts, enabling them to interact with both types of compounds. Since they simultaneously encounter unfavorable interactions, they may spontaneously self-organize into nanostructures to minimize their free energy. This process creates domains of different polarities, enabling solubilization of the nanostructure itself in the bulk medium and solubilization of a solute with opposite polarity. The resulting systems are not molecular solutions but colloidal dispersions, consisting of a dispersed phase or pseudo-phase (containing the solute), a dispersion medium (the solvent), and an interface between them. Their particle sizes range from about 1 to 1000 nm, placing colloidal dispersions between molecular solutions and coarse suspensions. Their high specific surface area imparts unique physical and chemical

properties. The nature of the amphiphile determines the architecture formed, its interface, and thus its stability against sedimentation or coalescence, depending on whether the solutes are solid or liquid. The processes involved and their influencing factors are a central topic in interface and colloid science³.

The most classic amphiphiles are surfactants, known as emulsifiers or detergents in industrial and household contexts. Their ability to self-assemble into various nanoarchitectures depending on the concentration is an inherent property⁴. Hydrotropes represent another group of amphiphilic compounds that do not form defined structures but instead self-organize around solutes⁵. Their solubilization mechanisms and systems formed in combination with water and oil are central to

Chapter 4 of this thesis.

Another type of structure formation driven by amphiphilicity involves proteins. These biopolymers consist of amino acids with varying polarities and create nanoarchitectures, known as protein conformations, through intra- and intermolecular interactions with their monomer side chains. Unlike surfactants, the type of nanoarchitecture they adopt, known as the native state, is predetermined by their functions as catalysts, transporters, signal integrators, and more. Protein folding is also guided by the minimization of free energy, but is highly sensitive to external stimuli. Changes in their inherent conformation are known as protein denaturation. Although denaturation causes a loss of specific functions, some properties related to their amphiphilic structure remain or can be restored⁶. Proteins are often used as emulsifiers and delivery systems in the food and pharmaceutical industries. As the trend toward a more plant-based diet grows, research on plant-based proteins has increased recently^{7,8}. The potential and limitations of industrially extracted plant proteins and their refunctionalization are the subject of **Chapter 2** of this thesis, with a focus on their water solubility. In **Chapter 3**, the application of plant proteins as solubilizers for the polyphenol curcumin is studied.

The combination of fundamental knowledge of solubilization mechanisms in the context of colloidal self-organization and a focus on sustainable formulations forms the central theme of this work. By systematically examining the water solubility of plant proteins, their potential to enhance curcumin solubility in water, and the interactions between surface-active compounds and surfactant-free microemulsions, this study aims to make an application-oriented contribution to sustainable formulation strategies.

1.2. Methodology

Spectroscopic, thermal, chromatographic, and electrokinetic methods were used to characterize the studied systems. In the following section, a comparative overview of the fundamental principles of the most relevant methods is provided.

1.2.1. Separation and Surface Analysis Techniques for Protein Characterization

Proteins are complex biopolymers, and their structures are sensitive to environmental conditions. Protein analysis includes a variety of different methods. From a colloidal perspective, the size distribution and surface properties in solution are essential parameters.

Size-exclusion chromatography (**SEC**) separates proteins (or aggregates) based on their molecular weight. Following basic principles of chromatography, SEC involves a mobile phase and a stationary phase. Unlike other chromatography techniques, the stationary phase in SEC is made of inert material with specific pore sizes, reducing adsorption. Separation is thus not enthalpy-driven by analyte-stationary phase interactions, but entirely entropy-driven. Proteins larger than these pores do not interact with the stationary phase and move with the mobile phase. Proteins within the pore size range enter the porous material and exhibit higher retention volumes. The separation based on molecular weight is largely linear, and absolute values can be estimated through calibration with proteins of known molecular weights. Detectors using various principles, such as viscosimetry, light scattering, or UV-Vis absorption, allow visualization of the separated fractions. By choosing suitable buffers for the mobile phase, gentle conditions provide a general overview of the sample's size distribution with minimal impact on molecular structure^{9–11}.

In discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (**SDS-PAGE**), proteins are also separated based on their molecular weight. Unlike in SEC, proteins are not separated in their native state; instead, negatively charged protein complexes form with the surfactant sodium dodecyl sulfate (SDS)^{12–14}. Complexation with SDS disrupts hydrophobic interactions within the protein, leading to denaturation^{15–17}. The amount of SDS bound to each protein correlates with the length of its polypeptide chain, resulting in an almost linear charge-to-mass ratio. These protein complexes are loaded onto a discontinuous polyacrylamide gel comprising a stacking gel and a separating gel. The gels differ in pore size, pH, and buffer composition. When an electric field is applied, the negatively charged complexes migrate toward the anode. The conditions in the stacking gel cause proteins to concentrate into a narrow band, a process called stacking. Upon entering the separating gel, the conditions change, and proteins migrate according to their molecular weight, with larger protein

complexes migrating more slowly than smaller ones. Including molecular weight standards enables the estimation of the apparent molecular weights of the separated proteins. Non-covalently linked structural motifs dissociate upon the addition of SDS. Covalent disulfide bridges can be disrupted by the addition of reducing agents, which is then known as reducing SDS-PAGE^{12–14}. While SEC reveals the (native) size distribution and oligomeric state of proteins, SDS-PAGE complements this by providing detailed insights into their subunit composition and molecular weights.

The **zeta potential** (ζ) describes the electrokinetic potential at the slipping plane of a particle. The absolute value of ζ indicates colloidal (in)stability, reflecting the electrostatic repulsion between charged particles in a dispersion. Following the Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory, larger absolute values of ζ indicate greater colloidal stability. In proteins, ζ results from the ionization of charged amino acid residues and interactions with ions in the surrounding solution. It is therefore strongly influenced by the medium's pH and ionic strength. There are several methods to determine ζ . One standardized approach is electrophoretic light scattering, which involves detecting the movement of charged protein particles in an electric field by measuring the Doppler shift of scattered laser light. The velocity of the particles is proportional to their electrophoretic mobility^{18–22}.

$$v_e = u_e \cdot E \quad (1.1)$$

with v_e as the particle velocity, u_e as the electrophoretic mobility, and E as the applied electric field. ζ can be calculated from u_e using the Helmholtz-Smoluchowski equation^{20,21}:

$$u_e = \frac{\varepsilon_{rs}\varepsilon_0\zeta}{\eta} \quad (1.2)$$

with ε_{rs} as the relative permittivity of the sample, ε_0 as the electric permittivity of vacuum, and η as the dynamic viscosity of the sample.

Depending on the ratio between particle size and the thickness of the electric double layer, different terms are added to the equation to describe ζ more accurately. The Helmholtz-Smoluchowski equation applies to thin electric double layers relative to the particle size. Since ζ cannot be measured directly and is highly influenced by measurement conditions, the reliability of ζ results depends on the chosen calculation model and measurement setup^{18–22}. Unlike SDS-PAGE, where the intrinsic charge of proteins is masked by SDS binding and separation depends on molecular size, ζ measurement allows the assessment of native electrostatic properties. When combined with SEC, ζ can also help interpret aggregation phenomena by providing information on charge-related interactions.

1.2.2. Optical Absorption and Emission Spectroscopy

Absorption of light in the UV-Vis region (200-750 nm) of the electromagnetic spectrum causes electronic excitation from the chromophor's ground state S_0 to an excited state S_1 . The resulting absorption spectra provide information on electronic transitions in the chromophore, the presence of optically superimposed absorption contributions, environmental conditions of the chromophore, such as interactions with the solvent or other chromophores, and enable the quantification of the chromophore's concentration. The information is obtained by accessing the difference between incident radiation intensity I_0 and the measured one I_A :

$$I_A = I_0 \cdot e^{-\varepsilon cd} \quad (1.3)$$

with ε as the molar absorption coefficient, c as the chromophor's concentration, and d as the thickness of the measuring cell.

The absorption A and the concentration c can then be calculated by the Lambert-Beer law:

$$A = \log \frac{I_0}{I_A} = \varepsilon \cdot c \cdot d \quad (1.4)$$

This linear relationship is valid only for dilute solutions with $c(\text{chromophor}) \leq 10^{-2}$ M. The return from S_1 to S_0 can occur via radiation and radiationless deactivation processes. **Fluorescence** is one possible radiation-based return to S_0 . The fluorescence intensity I_F is proportional to I_0 , the absorption, and the quantum yield ϕ :

$$I_F \propto \phi \cdot I_0 \cdot \varepsilon \cdot c \cdot d \cdot \text{instrumental factors} \quad (1.5)$$

$$\phi = \frac{k_F}{k_F + \sum k_{nr}} = k_F \cdot \tau_0 \quad (1.6)$$

with k_F as the rate constant of radiative processes, $\sum k_{nr}$ as the sum of rate constants of non-radiative processes, and τ_0 as the fluorescence lifetime^{23,24}.

I_F is thus influenced not only by the nature of the molecule and the sample composition but also by the instrumental setup. The intrinsic molecular properties ϕ and τ_0 are sensitive to the surrounding medium and provide further insight into the excited state and its behavior within it. Fluorescence spectroscopy is significantly more sensitive than UV-Vis absorption spectroscopy because it detects the emission of newly generated photons whose wavelength differs from that of the excitation radiation, rather than measuring small differences in the intensities of incident and transmitted light. This allows fluorescence to be detected with almost no background noise and the signal to be significantly amplified using highly sensitive detectors. This sensitivity advantage offers additional analytical value as fluorescence signals react strongly to changes in the molecular environment and

provide information on polarity, interactions, dynamics, and structure. Both methods are non-invasive and, in combination, provide profound information on a molecule's electronic structure and its interaction with its surrounding medium.

1.2.3. Scattering Methods

Scattering methods are based on the change in direction and intensity distribution of incident radiation caused by elastic or inelastic interactions with the medium's inhomogeneous density distributions. The scattering intensity I_S can be described analogously to that of absorption^{25,26}:

$$I_S = I_0 \cdot e^{-\tau d} \quad (1.7)$$

with τ as the turbidity.

Scattering methods are essential tools for non-invasive structural analysis of colloidal dispersions. By examining how the scattering intensity varies with angle or time correlation, details about the size, shape, spatial arrangements, and dynamics of the scatterers can be revealed. The type of radiation used determines the system requirements for successful measurements, the amount of information that can be obtained, and the resolution. The most common types of radiation used to study colloidal dispersions are light, X-rays, and neutrons. The size ranges of scattering objects and the suitable analysis techniques are summarized in Figure 1.1. While visible light is scattered due to differences in refractive indices between the particle and the surrounding medium, X-rays and neutrons are scattered by electrons and nuclei, respectively. To ensure reliable measurements, the difference between the particle and the surrounding medium, known as the contrast, must be sufficiently pronounced^{25–28}.

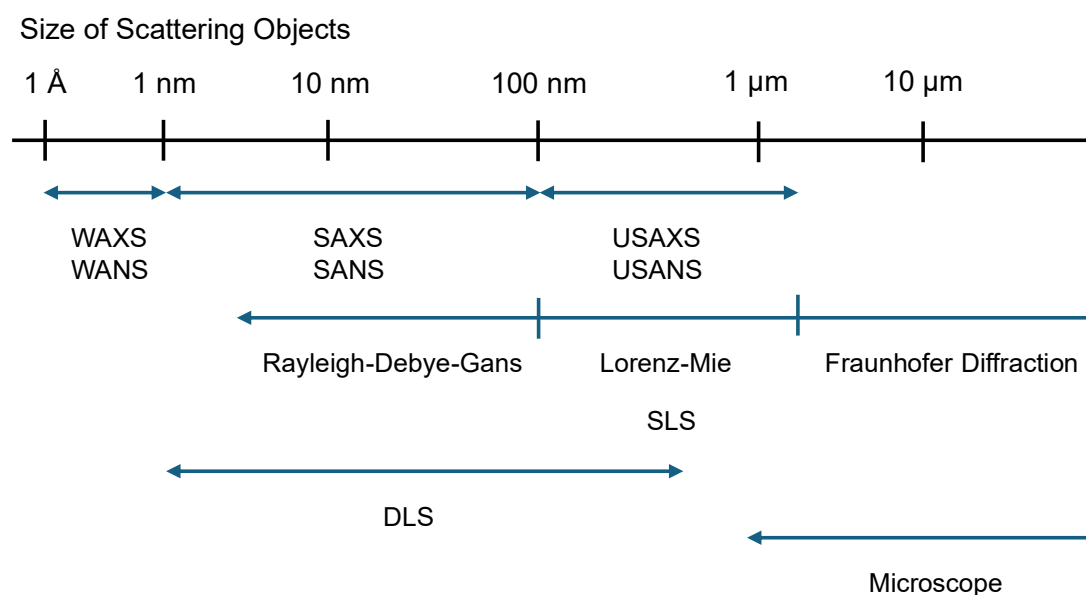


Figure 1.1: Size range of scattering objects and suitable scattering techniques for analysis, such as wide, small, and ultra small angle scattering of X-rays and neutrons (WAXS, SAXS, USAXS and WANS, SANS, USANS, respectively), various static light scattering (SLS) methods, dynamic light scattering (DLS), and microscopy. The figure is inspired by O. Glatter²⁸.

Two well-established methods for measuring particle size through light scattering are dynamic light scattering (DLS)^{25,29–34} and nanoparticle tracking analysis (NTA)^{35–39}. Both rely on the scattering of monochromatic, coherent laser light by particles undergoing Brownian motion in a liquid and provide similar information^{25,37}.

In **DLS**, the time dependence of scattering intensity fluctuations caused by the destructive and constructive interference of many moving scattering centers is measured simultaneously. The measurement setup is illustrated schematically in Figure 1.2. The laser beam hits the sample in a temperature-controlled cylindrical measurement cell, and a detector, usually a photomultiplier tube, records the scattered intensity as a function of the angle between the laser/sample and the detector. An autocorrelator processes the resulting Doppler shift spectrum into a normalized autocorrelation function $g^2(\tau)$ based on the theory of noise via Fourier transformation^{25,33}:

$$g^2(\tau) = \frac{\langle I(t) \cdot I(t + \tau) \rangle}{\langle |I(t)|^2 \rangle} \quad (1.8)$$

with $I(t)$ and $I(t + \tau)$ as the intensities at time t and after a certain decay time τ , respectively.

The diffusion coefficient, and thus particle size, can be determined from the field correlation function $g^1(\tau)$, which is described in equation 1.10, assuming monodisperse spherical aggregates. The Siegert relation (see equation 1.9) relates both correlation functions^{30,33}:

$$g^2(\tau) - 1 = \beta \cdot |g^1(\tau)|^2 \quad (1.9)$$

with β as the spatial coherence factor.

$$g^1(\tau) = \exp(-\Gamma\tau) \quad (1.10)$$

$$\Gamma = Dq^2 \quad (1.11)$$

with Γ as the decay rate, D as the translational diffusion coefficient, and q as the scattering vector length.

Assuming spherical aggregates and the factors that influence q , D is described by the Stokes-Einstein equation^{25,29,30,33}:

$$D = \frac{k_B T}{6\pi\eta R_h} \quad (1.12)$$

with k_B as the Boltzmann constant, T as the temperature, η as the dynamic viscosity of the medium, and R_h as the hydrodynamic radius of the spherical particle.

Particle size and polydispersity index are typically obtained by fitting the autocorrelation function using cumulant analysis. However, this method only provides reliable results if the particle sizes follow a monomodal distribution^{25,29,30,33}. If the system is polydisperse, other analysis strategies are more

suitable. One such algorithm is CONTIN^{40,41}. Both approaches assume the presence of spherical particles with well-defined interfaces in the system. In the case of fluctuating systems without defined interfaces, autocorrelation functions should not be evaluated quantitatively; instead, the intercept of the autocorrelation function provides a qualitative statement about the fluctuations^{42,43}. To further examine aggregates lacking well-defined interfaces, static scattering methods, such as small-angle X-ray scattering (SAXS), should be applied.

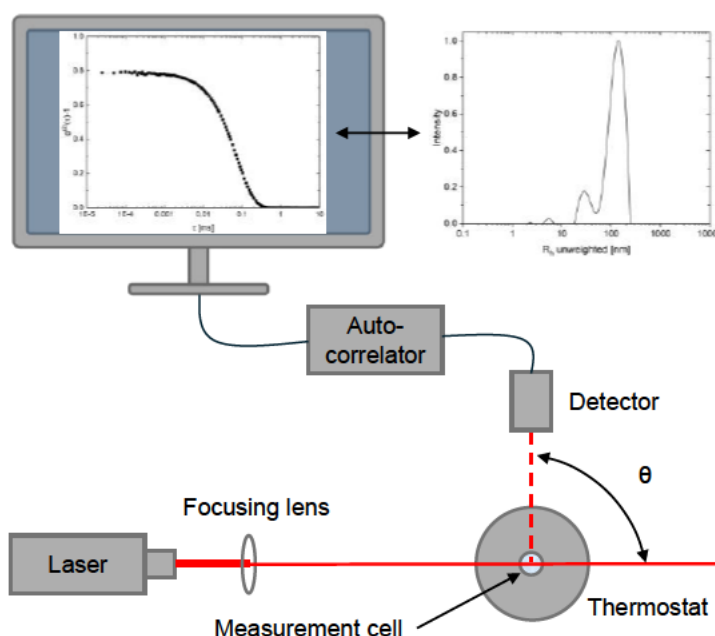


Figure 1.2: Schematic representation of the DLS measurement setup and the obtained information.

Particle size distributions from DLS are usually reported as intensity-weighted distributions. In the Rayleigh regime, where particles are much smaller than the wavelength of light, the scattered intensity scales with the sixth power of the particle diameter, causing larger particles or aggregates to dominate the measured signal even if they are in small quantities. For particles comparable to or larger than the wavelength (Lorenz-Mie regime), scattering becomes angle-dependent and is influenced by complex interference described by the form factor. Yet, larger particles still disproportionately influence the total intensity. This issue can be mitigated by converting intensity-weighted distributions into volume- or number-weighted distributions. However, these transformed distributions should be interpreted carefully, as they depend on model assumptions and can introduce additional uncertainties^{28,30,33}.

In NTA, the problem of overestimating large particles is eliminated because the method relies on optical tracking of individual particles³⁶. The schematic measurement principle is illustrated in Figure 1.3. A laser hits the sample at a low angle within a flow cell, and the moving particles scatter the incident light. An optical microscope visualizes the individual scattering centers, while a highly sensitive camera records their movement. The particles are tracked, and their trajectories are analyzed by image analysis software to determine each particle's diffusion coefficient using the two-dimensional Stokes-

Einstein relation (see equation 1.13), which then allows the calculation of each particle's hydrodynamic diameter. Unlike DLS, this method provides a size distribution weighted by number and enables concentration measurements^{35,38,39}.

$$\langle x, y \rangle^2 = \frac{k_B T}{3\pi\eta d_h} \quad (1.13)$$

with $\langle x, y \rangle^2$ as the mean-squared velocity of the particle and d_h as the hydrodynamic diameter.

Both methods have their advantages and disadvantages. In DLS, a bulk sample is analyzed, and particle size is determined by fitting the autocorrelation function. In NTA, on the other hand, the sample is examined at the single-particle level, with each movement evaluated individually. Consequently, the distributions tend to be more realistic, especially for polydisperse or multimodal samples. However, accuracy largely depends on camera focus, illumination settings, and the tracking algorithm, which introduces some user dependence. DLS provides fast and highly reproducible measurements. The measurement ranges of both methods overlap significantly (usually about 30-1000 nm), but the sensitive lower limit strongly depends on the particles' refractive index and on optical detection and camera resolution in NTA. Due to the measurement of a bulk phase in DLS, there is a lower detection limit of 2 nm, while in NTA it is around 30-50 nm^{35-37,39}.

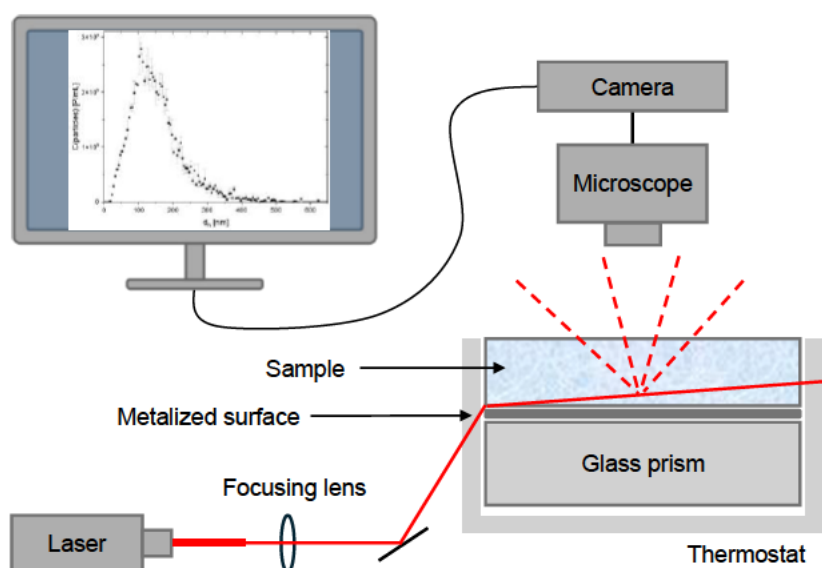


Figure 1.3: Schematic representation of the NTA measurement setup and the obtained information.

While DLS and NTA are based on analyzing light scattering caused by the movement of (individual) particles and mainly provide information about hydrodynamic size and size distribution, small-angle X-ray scattering (SAXS) is a static scattering method that offers details on shape, internal structure, and interscatterer interactions based on X-ray scattering^{26,28,44,45}. A schematic synchrotron SAXS measurement setup is illustrated in Figure 1.4. X-rays are deflected from the storage ring into the beamline. The collimated beam hits the sample, and a 2D-area detector captures the scattering

pattern. The distance between the sample and the detector depends on the range of interest of the scattering vector q . Its magnitude is given by:

$$q = \frac{4\pi}{\lambda} \sin \frac{\theta}{2} \quad (1.14)$$

with λ as the wavelength of the radiation and θ as the scattering angle.

The resulting 2D scattering pattern is reduced to a 1D scattering profile with the scattering intensity $I(q)$ plotted against the scattering vector q . For isotropically distributed scatterers, $I(q)$ is defined as:

$$I(q) = \Phi \cdot v \cdot \Delta\rho^2 \cdot S(q) \cdot P(q) + I(\text{background}) \quad (1.15)$$

with Φ as the volume fraction of the scatterers, v as the volume of the scatterer, $\Delta\rho^2$ as the contrast, $S(q)$ as the structure factor, $P(q)$ as the form factor, and $I(\text{background})$ as the scattering intensity from the background, such as solvent and capillary. The first three parameters do not depend on q and are summarized as forward scattering $I(0) = \Phi \cdot v \cdot \Delta\rho^2$. $\Delta\rho^2$ is calculated from the difference in scattering length densities, which are based on the electron densities between the scatterer and the surrounding medium. The structure factor $S(q)$ provides information on inter-scatterer interactions. The form factor $P(q)$ depends on the scatterer's shape. The scattering patterns are evaluated by fitting them to models containing the parameters of interest^{26,27,44–46}. The models used in this thesis are explained in the Experimental section of Chapter 4.

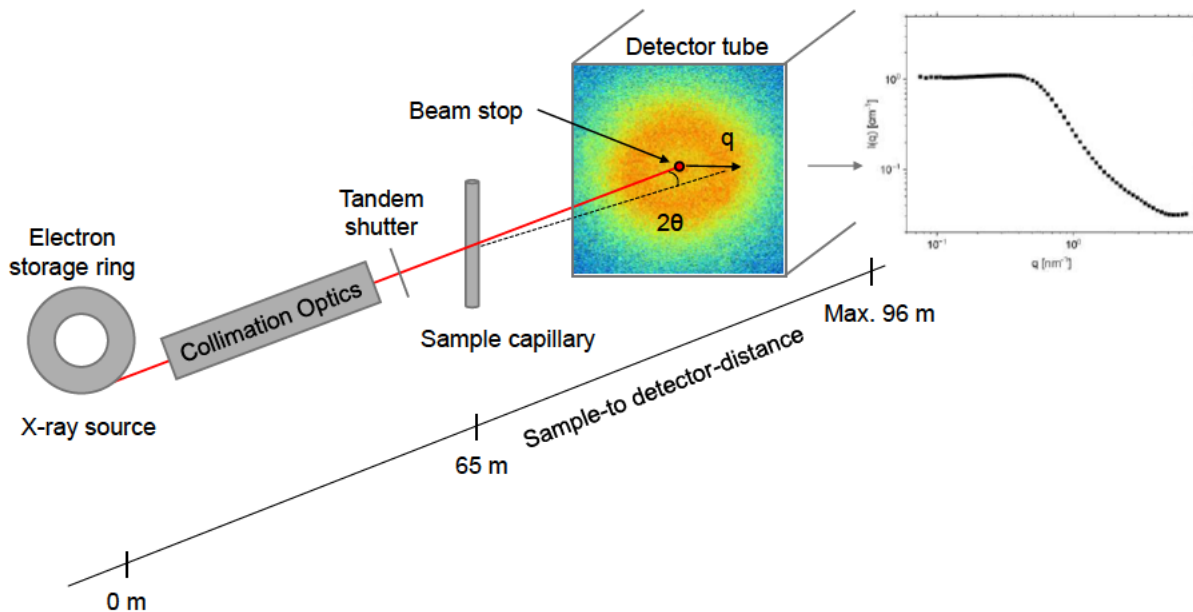


Figure 1.4: Schematic representation of the synchrotron SAXS measurement setup and the obtained information. Details on the setup and the distances is taken from information on the ID02 beamline at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France⁴⁷.

1.3. References

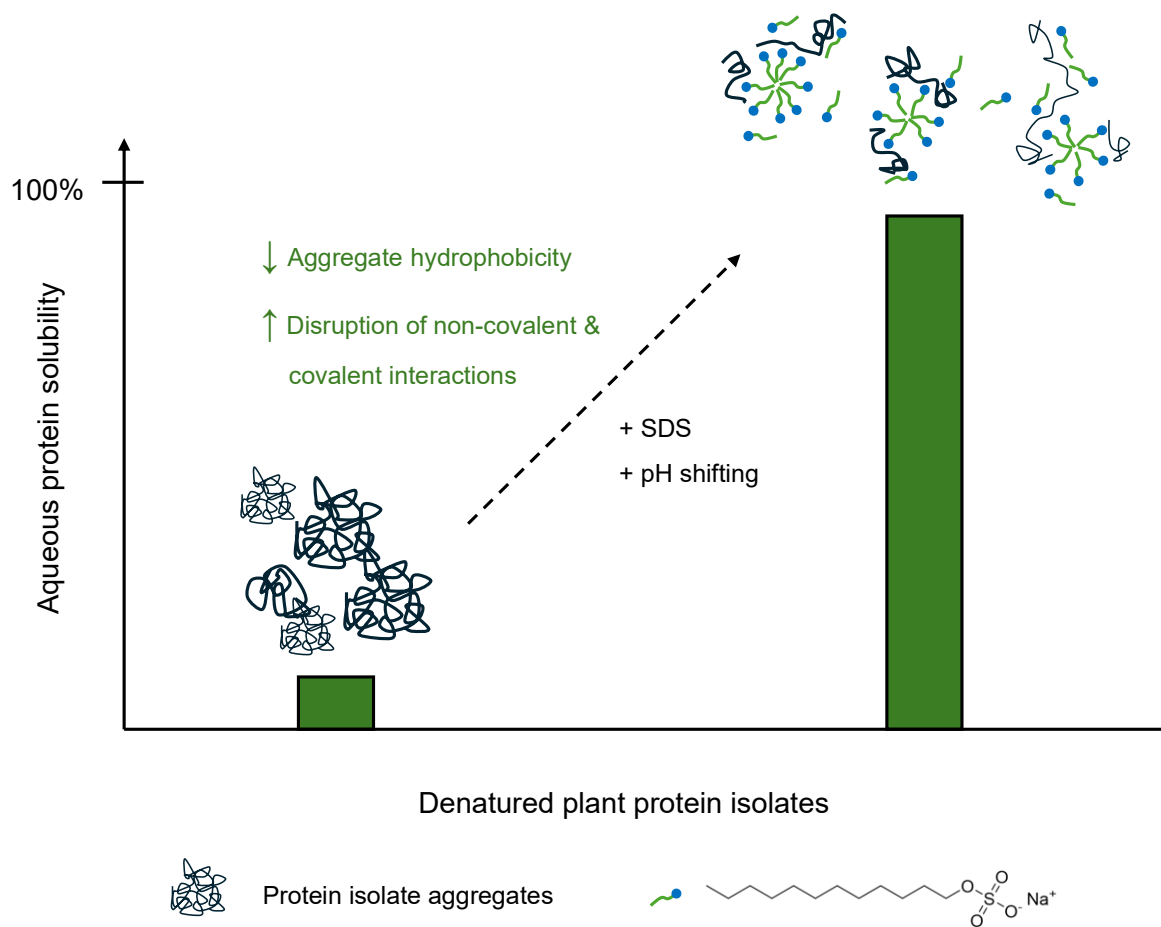
- (1) Nikolakakis, I.; Partheniadis, I. Self-Emulsifying Granules and Pellets: Composition and Formation Mechanisms for Instant or Controlled Release. *Pharmaceutics* **2017**, *9*, 50-77. <https://doi.org/10.3390/pharmaceutics9040050>.
- (2) Brower, V. Nutraceuticals: Poised for a healthy slice of the healthcare market? *Nat. Biotechnol.* **1998**, *16*, 728–731. <https://doi.org/10.1038/nbt0898-728>.
- (3) Evans, D. F.; Wennerström, H. *The Colloidal Domain: Where Physics, Chemistry, Biology, and Technology Meet*, second edition; Wiley-VCH: Weinheim, **1999**. ISBN 978-0-471-24247-5.
- (4) Holmberg, K. *Handbook of Applied Surface and Colloidal Chemistry*, Volume 2; John Wiley & Sons: Chichester, **2002**. ISBN 978-0-471-49083-8.
- (5) Kunz, W.; Holmberg, K.; Zemb, T. Hydrotropes. *Curr. Opin. Colloid Interface Sci.* **2016**, *22*, 99–107. <https://doi.org/10.1016/j.cocis.2016.03.005>.
- (6) Alberts, B.; Heald, R.; Johnson, A.; Morgan, D.; Raff, M.; Roberts, K.; Walter, P. *Molecular Biology of the Cell*, seventh edition; W. W. Norton & Company: New York, **2022**. ISBN 978-0-393-42708-0.
- (7) Gomes, A.; do Amaral Sobral, P. J. Plant Protein-Based Delivery Systems: An Emerging Approach for Increasing the Efficacy of Lipophilic Bioactive Compounds. *Molecules* **2021**, *27*, 60–90. <https://doi.org/10.3390/molecules27010060>.
- (8) Hadidi, M.; Tan, C.; Assadpour, E.; Kharazmi, M. S.; Jafari, S. M. Emerging plant proteins as nanocarriers of bioactive compounds. *J. Control. Release* **2023**, *355*, 327–342. <https://doi.org/10.1016/j.jconrel.2023.01.069>.
- (9) Barth, H. G.; Boyes, B. E.; Jackson, C. Size Exclusion Chromatography. *Anal. Chem.* **1996**, *68*, 445–466. <https://doi.org/10.1021/ac00084a022>.
- (10) Fekete, S.; Beck, A.; Veuthey, J.-L.; Guilleme, D. Theory and practice of size exclusion chromatography for the analysis of protein aggregates. *J. Pharm. Biomed. Anal.* **2014**, *101*, 161–173. <https://doi.org/10.1016/j.jpba.2014.04.011>.
- (11) Hong, P.; Koza, S.; Bouvier, E. S. P. Size-Exclusion Chromatography for the Analysis of Protein Biotherapeutics and their Aggregates. *J. Liq. Chromatogr. Relat. Technol.* **2012**, *35*, 2923–2950. <https://doi.org/10.1080/10826076.2012.743724>.
- (12) Smith, B. J. SDS Polyacrylamide Gel Electrophoresis of Proteins. In *Basic Protein and Peptide Protocols*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, **1994**; pp 23–34. <https://doi.org/10.1385/0-89603-268-x:23>.
- (13) Nowakowski, A. B.; Wobig, W. J.; Petering, D. H. Native SDS-PAGE: high resolution electrophoretic separation of proteins with retention of native properties including bound metal ions. *Metallomics* **2014**, *6*, 1068–1078. <https://doi.org/10.1039/c4mt00033a>.
- (14) Gallagher, S. R. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). *Curr. Protoc. Essent. Lab. Tech.* **2012**, *6*, 7.3.1-7.3.28. <https://doi.org/10.1002/9780470089941.et0703s06>.

- (15) Liu, K.; Hsieh, F.-H. Protein-protein interactions during high-moisture extrusion for fibrous meat analogues and comparison of protein solubility methods using different solvent systems. *J. Agric. Food Chem.* **2008**, *56*, 2681–2687. <https://doi.org/10.1021/jf073343q>.
- (16) Martin, A. H.; Nieuwland, M.; Jong, G. A. H. de. Characterization of Heat-Set Gels from RuBisCO in Comparison to Those from Other Proteins. *J. Agric. Food Chem.* **2014**, *62*, 10783–10791. <https://doi.org/10.1021/jf502905g>.
- (17) Otzen, D. E.; Pedersen, J. N.; Østergaard Rasmussen, H.; Pedersen, J. S. How do surfactants unfold and refold proteins? *Adv. Colloid Interface Sci.* **2022**, *308*, 102754–102767. <https://doi.org/10.1016/j.cis.2022.102754>.
- (18) Serrano-Lotina, A.; Portela, R.; Baeza, P.; Alcolea-Rodriguez, V.; Villarroel, M.; Ávila, P. Zeta potential as a tool for functional materials development. *Catal. Today* **2023**, *423*, 113862–113873. <https://doi.org/10.1016/j.cattod.2022.08.004>.
- (19) Jacobasch, H.-J. Characterization of solid surfaces by electrokinetic measurements. *Prog. Org. Coatings* **1989**, *17*, 115–133. [https://doi.org/10.1016/0033-0655\(89\)80018-4](https://doi.org/10.1016/0033-0655(89)80018-4).
- (20) Delgado, A. V.; González-Caballero, F.; Hunter, R. J.; Koopal, L. K.; Lyklema, J. Measurement and Interpretation of Electrokinetic Phenomena (IUPAC Technical Report). *Pure Appl. Chem.* **2005**, *77*, 1753–1805. <https://doi.org/10.1016/j.jcis.2006.12.075>.
- (21) Cano-Sarmiento, C.; Téllez-Medina, D. I.; Viveros-Contreras, R.; Cornejo-Mazón, M.; Figueroa-Hernández, C. Y.; García-Armenta, E.; Alamilla-Beltrán, L.; García, H. S.; Gutiérrez-López, G. F. Zeta Potential of Food Matrices. *Food Eng. Rev.* **2018**, *10*, 113–138. <https://doi.org/10.1007/s12393-018-9176-z>.
- (22) Salgin, S.; Salgin, U.; Bahadır, S. Zeta Potentials and Isoelectric Points of Biomolecules: The Effects of Ion Types and Ionic Strengths. *Int. J. Electrochem. Sci.* **2012**, *7*, 12404–12414. [https://doi.org/10.1016/S1452-3981\(23\)16554-0](https://doi.org/10.1016/S1452-3981(23)16554-0).
- (23) Atkins, P. W.; de Paula, J.; Keeler, J. J. *Physikalische Chemie*, 6. Auflage; Wiley-VCH: Weinheim, **2021**. ISBN 978-3-527-34550-2.
- (24) Hesse, M.; Meier, H.; Zeeh, B. *Spektroskopische Methoden in der organischen Chemie*, 8. Auflage; Georg Thieme Verlag KG: Stuttgart, **2014**. ISBN 978-3-13-160038-7.
- (25) Øgden, L. *Light Scattering Demystified: Theory and Practice*, **2017**.
- (26) Pusey, P. N. Introduction to Scattering Experiments. In *Neutrons, X-rays, and Light: Scattering Methods Applied to Soft Condensed Matter*, first edition; Lindner, P., Zemb, T., Eds.; Elsevier: Amsterdam, **2002**; pp 3–21. ISBN 978-0-444-51122-5.
- (27) Spalla, O. General Theorems in Small-Angle Scattering. In *Neutrons, X-rays, and Light: Scattering Methods Applied to Soft Condensed Matter*, first edition; Lindner, P., Zemb, T., Eds.; Elsevier: Amsterdam, **2002**; pp 49–71. ISBN 978-0-444-51122-5.
- (28) Glatter, O. Static Light Scattering of Large Systems. In *Neutrons, X-rays, and Light: Scattering Methods Applied to Soft Condensed Matter*, first edition; Lindner, P., Zemb, T., Eds.; Elsevier: Amsterdam, **2002**; pp 171–201. ISBN 978-0-444-51122-5.
- (29) Pecora, R. Dynamic Light Scattering Measurement of Nanometer Particles in Liquids. *J. Nanopart. Res.* **2000**, *2*, 123–131. <https://doi.org/10.1023/A:1010067107182>.

- (30) Stetefeld, J.; McKenna, S. A.; Patel, T. R. Dynamic light scattering: a practical guide and applications in biomedical sciences. *Biophys. Rev.* **2016**, *8*, 409–427. <https://doi.org/10.1007/s12551-016-0218-6>.
- (31) Nicolai, T.; Brown, W.; Johnsen, R. M.; Stepanek, P. Dynamic behavior of θ solutions of polystyrene investigated by dynamic light scattering. *Macromolecules* **1990**, *23*, 1165–1174. <https://doi.org/10.1021/ma00206a040>.
- (32) Hoo, C.; Starostin, N.; West, P.; Mecartney, M. A comparison of atomic force microscopy (AFM) and dynamic light scattering (DLS) methods to characterize nanoparticle size distribution. *J. Nanopart. Res.* **2008**, *10*, 89–96. <https://doi.org/10.1007/s11051-008-9435-7>.
- (33) Hassan, P. A.; Rana, S.; Verma, G. Making sense of Brownian motion: colloid characterization by dynamic light scattering. *Langmuir* **2015**, *31*, 3–12. <https://doi.org/10.1021/la501789z>.
- (34) Gugliotta, L. M.; Vega, J. R.; Meira, G. R. Latex Particle Size Distribution by Dynamic Light Scattering: Computer Evaluation of Two Alternative Calculation Paths. *J. Colloid Interface Sci.* **2000**, *228*, 14–17. <https://doi.org/10.1006/jcis.2000.6923>.
- (35) Dragovic, R. A.; Gardiner, C.; Brooks, A. S.; Tannetta, D. S.; Ferguson, D. J. P.; Hole, P.; Carr, B.; Redman, C. W.; Harris, A. L.; Dobson, P. J.; *et al.* Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. *NBM* **2011**, *7*, 780–788. <https://doi.org/10.1016/j.nano.2011.04.003>.
- (36) Filipe, V.; Hawe, A.; Jiskoot, W. Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. *Pharm. Res.* **2010**, *27*, 796–810. <https://doi.org/10.1007/s11095-010-0073-2>.
- (37) Chan, M. Y.; Dowling, Q. M.; Sivananthan, S. J.; Kramer, R. M. Particle Sizing of Nanoparticle Adjuvant Formulations by Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA). In *Vaccine Adjuvants: Methods and Protocols*; Fox, C. B., Ed.; Springer: New York, **2017**; pp 239–252. https://doi.org/10.1007/978-1-4939-6445-1_17.
- (38) Hole, P.; Sillence, K.; Hannell, C.; Maguire, C. M.; Roesslein, M.; Suarez, G.; Capracotta, S.; Magdolenova, Z.; Horev-Azaria, L.; Dybowska, A.; *et al.* Interlaboratory comparison of size measurements on nanoparticles using nanoparticle tracking analysis (NTA). *J. Nanopart. Res.* **2013**, *15*, 2101–21013. <https://doi.org/10.1007/s11051-013-2101-8>.
- (39) Kowkabany, G.; Bao, Y. Nanoparticle Tracking Analysis: An Effective Tool to Characterize Extracellular Vesicles. *Molecules* **2024**, *29*, 4672–4689. <https://doi.org/10.3390/molecules29194672>.
- (40) Provencher, S. W. CONTIN: A general purpose constrained regularization program for inverting noisy linear algebraic and integral equations. *Comput. Phys. Commun.* **1982**, *27*, 229–242. [https://doi.org/10.1016/0010-4655\(82\)90174-6](https://doi.org/10.1016/0010-4655(82)90174-6).
- (41) Provencher, S. W. A constrained regularization method for inverting data represented by linear algebraic or integral equations. *Comput. Phys. Commun.* **1982**, *27*, 213–227. [https://doi.org/10.1016/0010-4655\(82\)90173-4](https://doi.org/10.1016/0010-4655(82)90173-4).
- (42) Buchecker, T.; Krickl, S.; Winkler, R.; Grillo, I.; Bauduin, P.; Touraud, D.; Pfitzner, A.; Kunz, W. The impact of the structuring of hydrotropes in water on the mesoscale solubilisation of a

- third hydrophobic component. *Phys. Chem. Chem. Phys.* **2017**, *19*, 1806–1816. <https://doi.org/10.1039/C6CP06696H>.
- (43) Krickl, S.; Touraud, D.; Bauduin, P.; Zinn, T.; Kunz, W. Enzyme activity of horseradish peroxidase in surfactant-free microemulsions. *J. Colloid Interface Sci.* **2018**, *516*, 466–475. <https://doi.org/10.1016/j.jcis.2018.01.077>.
- (44) Li, T.; Senesi, A. J.; Lee, B. Small Angle X-ray Scattering for Nanoparticle Research. *Chem. Rev.* **2016**, *116*, 11128–11180. <https://doi.org/10.1021/acs.chemrev.5b00690>.
- (45) Sivia, D. S. *Elementary Scattering Theorie: For X-ray and Neutron Users*; Oxford University Press: Oxford, **2011**. ISBN 978-0199228676.
- (46) Naskar, B.; Diat, O.; Nardello-Rataj, V.; Bauduin, P. Nanometer-Size Polyoxometalate Anions Adsorb Strongly on Neutral Soft Surfaces. *J. Phys. Chem. C* **2015**, *119*, 20985–20992. <https://doi.org/10.1021/acs.jpcc.5b06273>.
- (47) Narayanan, T.; Sztucki, M.; Zinn, T.; Kieffer, J.; Homs-Puron, A.; Gorini, J.; van Vaerenbergh, P.; Boesecke, P. Performance of the time-resolved ultra-small-angle X-ray scattering beamline with the Extremely Brilliant Source. *J. Appl. Crystallogr.* **2022**, *55*, 98–111. <https://doi.org/10.1107/s1600576721012693>.

Chapter 2 Solubilization of Plant Proteins in Aqueous Solutions



Significant parts of this chapter were conducted in collaboration with the company dsm-firmenich. Tobias Boyke and Dominik Straub provided support in protein solubility experiments. Nadine Stefan and Prof. Dr. Rainer Müller performed SDS-PAGE and TGA, respectively, on my behalf.

2.1. Abstract

With growing awareness of health and environmental concerns, as well as the unequal global distribution of food, plant proteins have become increasingly significant in scientific, political, and societal debates. Replacing livestock protein with plant protein also involves techno-functional applications. Like animal proteins, plant proteins are functional compounds with an amphiphilic structure, providing solubility in water and oil, as well as gelling, emulsifying, and foaming properties. They are extracted from plant materials, often under denaturing conditions, which frequently results in the loss of desired functionalities, especially water solubility. In this chapter, a selection of commercially available plant protein isolates is characterized and modified to enhance their water solubility. By testing well-established physical and chemical modification methods and combining them in new sequences, a better understanding of the factors limiting the solubility of denatured protein aggregates was achieved. Experiments on pulse protein isolates from pea (PPIp) and chickpea (CPIp), and tuber proteins from potato (PoPIp) demonstrated that focusing on individual factors alone may not be sufficient to increase water solubility; instead, a combination of complementary factors is essential. Complete protein solubilization of all isolates was achieved within a single approach that combined the dual actions of sodium dodecyl sulfate (SDS) with proteins and pH shifting: SDS interacts with proteins through hydrophobic interactions and acts as a surfactant. pH shifting induces electrostatic repulsion in protein aggregates, causing them to loosen or disassemble. It was concluded that for commercial protein isolates, the key to restoring solubility lies in the interplay between reducing the protein's hydrophobicity and disrupting processing- and extraction-induced aggregates. Particle size and zeta potential of the selected modifications were analyzed, and a schematic diagram of the solubilization mechanism is provided.

2.2. Fundamental Information

2.2.1. Protein Structure and Stability

Proteins are the functional molecules of all vital processes. They are biopolymers composed of α -amino acids linked together by peptide bonds. This fundamental structural motif was first proposed in 1882 by the French chemist E. Grimaux and was later presented independently by Emil Fischer and Franz Hofmeister at the 74th meeting of the Society of German Scientists and Physicians in Karlsbad, Germany, in 1902¹. The unbranched chain of different α -amino acids, known as the *primary structure*, forms the basis of the conformation and functionality of every protein. Non-covalent interactions (hydrogen bonds, electrostatic interactions, van der Waals attractions, and hydrophobic clustering forces) between amino acids in the primary structure create an intramolecular spatial arrangement, leading to the formation of beta sheets and helices. These structural motifs are called *secondary structures*. Secondary structures arrange themselves three-dimensionally. This higher-order organization is called the *tertiary structure*. If a protein is encoded with more than one polypeptide chain, the built-up tertiary structure subunits thereof assemble spatially and form the *quaternary structure*². This scientific breakthrough was awarded the Nobel Prize in 1962 to Max Ferdinand Perutz and John Cowdery Kendrew³. *Tertiary* and *quaternary structures* form through hydrogen bonds, disulfide bonds, salt bridges, cation- π , and hydrophobic interactions⁴⁻⁷. To function as building blocks, catalysts, transporters, signal integrators, and more, proteins must possess a predetermined, intact tertiary structure (or an intact quaternary structure if multiple polypeptide chains are present). This state is known as the *native* state and is generally characterized by a hydrophobic core and a polar exterior.

Protein conformation is arranged according to the minimization of free energy. Yet, it is a dynamic process that responds sensitively to external stimuli such as thermal energy^{2,8}. The unfolding of the specific protein conformation is referred to as *denaturation*. Denaturation leads to destabilization and, consequently, de-functionalization of a protein². The different energy levels of classical protein conformations are schematically visualized in Figure 2.1. The unfolded state exhibits a high degree of hydration, resulting in high Gibbs free energy and conformational entropy. This conformer is the thermodynamically least favorable. Intramolecular contacts induce protein folding, while intermolecular contacts result in protein aggregation. Both interaction pathways reduce free energy, hydration, and conformational entropy. The degree of energy minimization, i.e., the degree of stabilization of different conformations, is illustrated by funnels of varying depths. Each folding state corresponds to a discrete point in the energy landscape. Thermodynamically preferred states are more

populated, which correlates with funnel narrowing, forming local minima that represent distinct conformations with low conformational entropy^{10–12}. The funnel also illustrates the delicate balance between the unfolded state and distinct conformations, enabling proteins to fluctuate between various conformations in response to external stimuli¹³. Not all pathways are reversible; some aggregation processes are irreversible and represented by deeper funnels¹⁴.

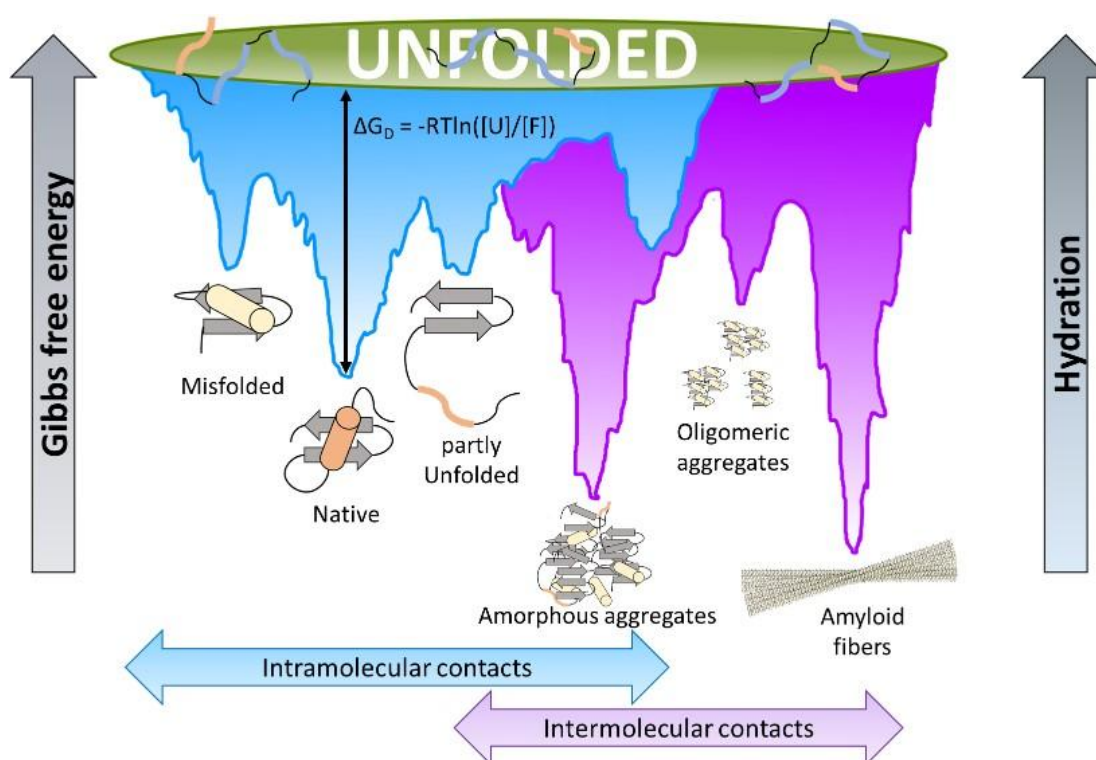


Figure 2.1: Energy landscape diagram of proteins in solution depicting the conformational space. Reprinted with permission from Johannes Mehringer⁹, who adapted the graphic from the literature^{10,11}.

Protein aggregation can be categorized according to three mechanisms: aggregation via unfolded conformations, aggregation resulting from protein self-association or chemical cross-linking, and aggregation induced by chemical degradation. All mechanisms lead to either amyloid fibrils or amorphous aggregates. While amyloid fibrils possess a highly ordered, linear cross- β structure, amorphous aggregates are highly disordered clusters comprised of unfolded proteins associated through their exposed hydrophobic regions⁸. Protein misfolding, driven by genetic or physiological triggers, leads to a group of human disorders. Among others, Creutzfeldt-Jakob disease, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, and Huntington's disease are classified as protein-folding diseases. The latter can be further summarized as amyloid diseases – proteins misfold irreversibly into insoluble amyloid fibrils, which then accumulate and deposit in the patient's tissues, causing neurodegeneration. Recognizing protein misfolding as a central factor in disease mechanisms is a significant achievement over the past 70 years. Current research focuses on understanding protein aggregation and exploring ways to rescue misfolded proteins^{8,10,11,13,15,16}.

2.2.2. Protein Solubility: Definition, Classification, and Influencing Parameters

2.2.2.1. Definition of Protein Solubility

Proteins are macromolecules that form colloidal solutions if their primary structure remains intact during solubilization^{17,18}. Protein solubility can be examined from both thermodynamic and kinetic perspectives: Thermodynamic solubility defines the equilibrium protein concentration in the solvent within a one- or two-phase system (liquid-solid) at a specific temperature and pressure, with the solid protein as the starting material for solubilization. In this context, the equilibrium protein concentration is called a saturation concentration. The equilibrium between the solubilized protein and the solid phase can be characterized by equal chemical potentials μ of both phases^{17,19,20}:

$$\mu_l = \mu_{l,s}^\circ + RT \ln(a_{l,s}) = \mu_s \quad (2.1)$$

with l and s as abbreviations for the liquid and solid phase, respectively, μ° as the chemical potential under ideal conditions, R as the gas constant, T as the temperature, and a as the activity of the protein.

Suppose the protein is pre-solubilized and then exposed to a solvent or conditions where its thermodynamic solubility is lower. In this case, a temporarily higher concentration than the equilibrium value is typically observed. The protein is kinetically solubilized. The resulting supersaturated solution exists in a non-equilibrium state, and the protein will precipitate or crystallize to some extent, according to nucleation theory. Supersaturation can be quantified as the ratio of the solubilized protein concentration to the equilibrium protein concentration. The difference between the two concentrations indicates the proportion of kinetic solubilization^{17,19}.

$$S = \frac{a_l}{a_l^*} \approx \frac{c_l}{c_l^*} \quad (2.2)$$

with a_l and c_l as the activity and concentration of the protein in solution, and a_l^* and c_l^* as the activity and concentration of the protein in thermodynamic equilibrium.

In the following, early approaches and current understanding of protein solubility and its influencing factors are discussed.

2.2.2.2. The Osborne Classification

Research into protein aggregation and solubility has been a focus of study even before the basic structure of proteins was uncovered. Early experiments date back to the 18th century and focused on the solubilization of wheat gluten. The influence of heat, acid, base, and various solvents on the solubility of plant proteins was studied in subsequent years. In 1909, the American biochemist Thomas B. Osborne published “The vegetable proteins”, a comprehensive overview of the knowledge of plant proteins. Although his studies focused on the isolation and purification of proteins from cereals, pulses, and seeds, their structural similarity to animal proteins known at the time allowed for meaningful comparisons. He compared their properties with those of animal proteins and proposed a solubility-based categorization of vegetable proteins, offering insights relevant to both protein types²¹. This categorization is known as the Osborne fractions and is still used today (see Table 2.1).

Table 2.1: Scheme of the solubility of vegetable proteins according to Thomas B. Osborne²¹.

Protein fraction	Suitable solvent
Albumins	Water
Globulins	Diluted salt solution
Prolamins	70% ethanolic solution
Glutelins	Acidic or basic solution

Temperature significantly impacts protein structure and is discussed in “The vegetable proteins” as a key parameter influencing protein solubility²¹. In a modern perspective, thermal denaturation refers to the loss of a protein's native structure due to changes in temperature. Protein conformation depends on the balance between intramolecular interactions and conformational entropy. The entropy contribution includes several components, many of which tend to increase with rising temperature: release of bound water (leading to solvent entropy gain), increased conformational freedom of the polypeptide chain during unfolding, changes in the entropy of mixing, and reorganization of the hydration shell. Increasing the temperature generally destabilizes the native structure because unfolded states are entropically favored. The temperature at which the entropic contribution first dominates intramolecular interactions is the thermal denaturation temperature. This leads to the exposure of hydrophobic and thiol groups, often followed by aggregation due to attractive intermolecular interactions. This process reduces protein solubility^{17,19}. Increasing the temperature below the denaturation temperature generally enhances protein solubility²².

2.2.2.3. The Impact of pH on Protein Solubility

Osborne's classification of vegetable proteins provided one of the earliest systematic approaches to protein fractionation. He also noted that the solubilization behavior is influenced not only by external conditions but also by protein denaturation²¹. However, since protein conformation had not yet been clarified, his work did not systematically consider the impact of environmental pH on protein charge states and structural dynamics. It was not until the 1920s that Leonor Michaelis and others introduced the concept of the isoelectric point (pI), linking the electrostatic properties of a protein to macroscopic phenomena like solubility, aggregation, and viscosity²³. The protonation state of ionizable amino acid side chains, and thus the protein's net charge, depends on environmental pH. This state is further influenced by the protein's conformation and its physicochemical environment, such as ionic strength. Proteins migrate according to their net charge when exposed to an electric field. The pH at which the net charge is zero and, thus, no migration in an electric field occurs, is defined as the isoelectric point (pI). As a result, proteins are prone to aggregation and precipitation at the pI^{24,25}. Knowing a protein's pI is a key parameter in modern protein analysis. When away from the pI, electrostatic repulsion between similarly charged side groups causes increased solubility. Additionally, the reactivity of proteins is enhanced due to exposure of the hydrophobic core and disruption of side chain interactions^{19,26}.

2.2.2.4. The Impact of Specific Ion Effects and Ionic Strength on Protein Solubility

At the same time, Osborne was performing his experiments, the German physician Franz Hofmeister and his co-workers were studying the solubility of animal proteins. They published seven papers in the 1880s and 1890s, summarized under the German title "Zur Lehre von der Wirkung der Salze". They examined how salts influence the solubility of hen egg globulins. Hofmeister found that strongly hydrated anions decreased the protein solubility (salting-out effect), while weakly hydrated anions increased it (salting-in effect)^{27,28}. This series was expanded to include cations and is today known as the Hofmeister series.

Kim D. Collins contributed to the understanding of the Hofmeister series by defining the interactions between ions and water, based on his research on ion pairing due to matching water affinities: salting-out anions with high charge density, known as *kosmotropes*, form stronger interactions with water than water does with itself, while salting-in anions with low charge density, known as *chaotropes*, have weaker interactions with water than water does with itself²⁹. The Hofmeister series, which includes some anions already studied by Hofmeister^{28,30} (marked in bold) and additional ones from later research³⁰⁻³², is presented in Figure 2.2. Anions are ordered by charge density. Although the exact

molecular mechanisms underlying the Hofmeister effects remain elusive, individual factors such as solute dehydration, preferential adsorption, volume exclusion, and dispersion interactions are considered key contributors^{29,33–37}. The concept was further extended to include amphiphilic molecules with a charged head group and a hydrophobic tail^{38–41}. Mehringer *et al.* proposed an extended Hofmeister-inspired series that considers the balance between the salting-in and salting-out behavior of the anionic headgroup and the hydrophobicity of the tail⁴².

Strongly hydrated High charge density	Transition	Weakly hydrated Low charge density
$\text{C}_3\text{H}_5\text{O}(\text{CO}_2)_3^{3-} < \text{C}_4\text{H}_4\text{O}_6^{2-} < \text{SO}_4^{2-} < \text{HPO}_4^{2-} < \text{CH}_3\text{CO}_2^- < \text{HCO}_3^- < \text{F}^- < \text{Cl}^- < \text{Br}^- < \text{NO}_3^- < \text{I}^- < \text{ClO}_3^- < \text{SCN}^-$		
Salting-out		Salting-in

Figure 2.2: The Hofmeister series arranged according to charge density. From left to right: Citrate, tartrate, sulphate, hydrogen phosphate, acetate, hydrogen carbonate, fluoride, chloride, bromide, nitrate, iodide, chlorate, and thiocyanate. Hofmeister already studied the ones marked in bold.

Apart from specific ion effects, the protein solubility is influenced by the ionic strength of a solution. Increasing ionic strength with the monovalent salt NaCl leads to the shielding of electrostatic interactions between protein molecules, as described by the Debye screening theory. This generally leads to a non-linear solubility pattern with alternating phases of salting-in and salting-out. At very low ionic strength (< 0.1 M), solubility is high due to strong electrostatic repulsions between proteins of the same charge. As ionic strength increases (0.1–0.5 M), solubility initially drops because screening of the electric double-layer diminishes the zeta potential and promotes aggregation. However, with further increases (0.5–1.0 M), surface charges can increase, thereby increasing solubility again. At very high salt concentrations (> 1 M), solubility decreases again due to reduced water availability for protein hydration. The extent of these effects depends strongly on the pH value and the protein structure. Particularly around the isoelectric point, solubility can be significantly increased by adding ions, as intramolecular charge interactions are effectively shielded^{19,22,43}.

2.2.2.5. The Impact of Additives on Protein Solubility

In addition to the classic influencing factors of pH, temperature, and ionic strength, low-molecular-weight additives can stabilize or destabilize protein structure. The molecular structures of the presented additives are depicted in Figure 2.3. Urea and guanidinium hydrochloride are chaotropic compounds that bind non-covalently to protein surfaces through hydrogen bonds (urea, guanidinium hydrochloride), electrostatic interactions, and π - π interactions (guanidinium hydrochloride), thereby promoting protein unfolding. At lower concentrations (0.5–2 M), denaturants prevent aggregation by solubilizing hydrophobic folding intermediates. With increasing denaturant concentration (up to 4–6 M), complete solubilization of proteins can be achieved^{44–47}. Sodium dodecyl sulfate (SDS) is an

anionic surfactant with a critical micellar concentration (CMC) of 8 mM⁴⁸. It is commonly used as a denaturant in SDS-PAGE to interrupt non-covalent interactions in proteins^{49–51}. Otzen *et al.* reviewed current research on the denaturation mechanism of SDS micelles on native proteins and summarized the following: The denaturation mechanism of SDS can be described as a core-shell model with SDS micelles surrounded by (partially) unfolded protein molecules. The mechanism involves electrostatic interactions between the charged sulfate headgroup of SDS and complementarily charged protein side chains. Additionally, hydrophobic interactions between hydrophobic alkyl chains and hydrophobic patches on the protein drive the association of SDS micelles with the protein, forming heterogeneous complexes. SDS micelles are stabilized by charge screening through interactions with complementarily charged groups on the protein. The interaction of the proteins with SDS, on the other hand, alters the electrostatic environment and the hydrogen bonding and, thus, the interaction forces between the protein's secondary, tertiary, and quaternary structures. Hydrophobic interactions dominate in the case of non-ionizable side groups⁴⁹. Dithiothreitol (DTT) and β -mercaptoethanol are reducing agents, reducing covalent disulfide bridges to sulfhydryl groups^{45,50}. By cleaving covalent bonds and exposing free sulfhydryl groups, solubility can be enhanced. All the presented additives modify the secondary and tertiary structures of proteins, while the primary structures remain intact.

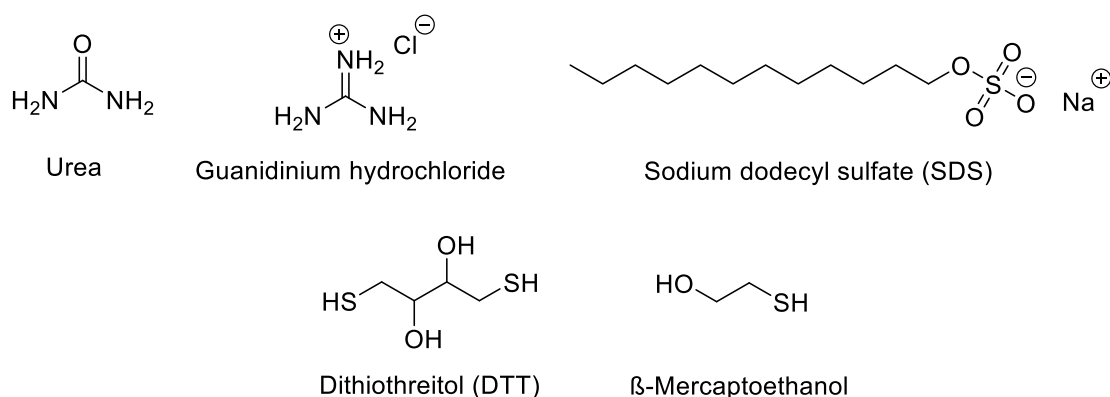


Figure 2.3: Molecular structures of the additives urea, guanidinium hydrochloride, sodium dodecyl sulfate (SDS), Dithiothreitol (DTT), and β -mercaptoethanol.

While intrinsic factors such as amino acid composition, hydrophobicity, and surface charge distribution fundamentally determine a protein's solubility, extrinsic parameters, including pH, temperature, and the addition of salts and other additives, can significantly influence this property¹⁷. These factors, as well as plant-related factors (discussed in more detail in sections 2.2.4.2 and 2.2.5.1) are summarized in Figure 2.4. Understanding how these external factors interact with the protein's inherent characteristics is crucial for comprehending protein solubility. The loss of solubility due to aggregation is often energetically favorable (see Figure 2.1), making it very difficult or impossible to re-solubilize after aggregation and precipitation. The relevance of re-solubilizing protein aggregates extends beyond the pathological context mentioned above. The intrinsic tendency of proteins to aggregate also presents challenges in applied protein science.

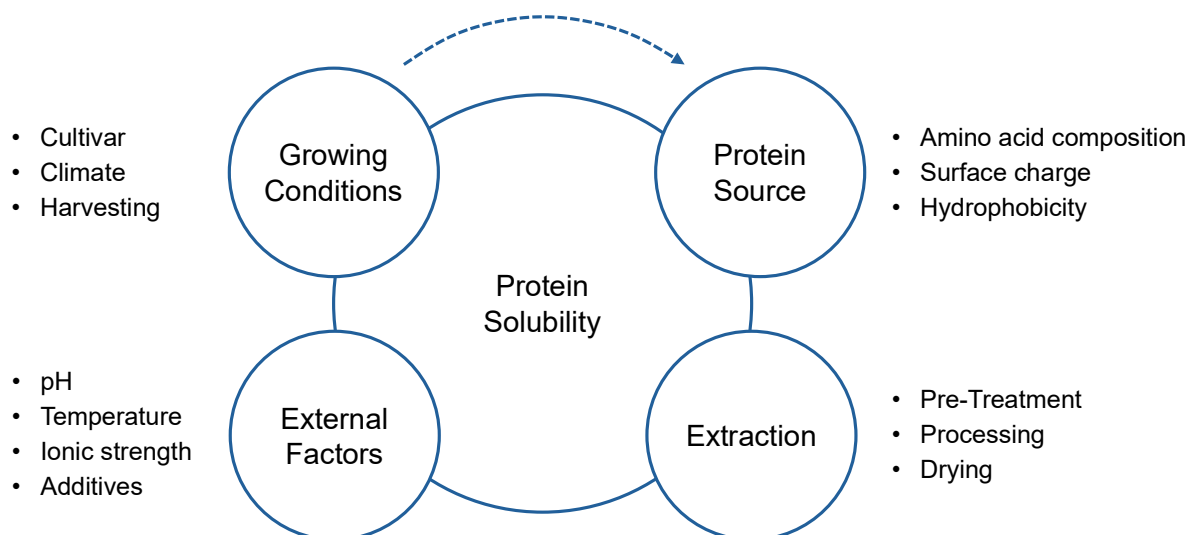


Figure 2.4: Influencing factors on protein solubility: growing conditions, protein source, extraction, and external factors.

The following sections analyze the differences between plant and animal proteins regarding global sustainability and nutritional value. The focus then shifts to re-functionalizing plant-based proteins for use in the food industry. It is important to note that 're-functionalization' refers to restoring techno-functional qualities, such as emulsifying ability, rather than returning to native protein structure.

2.2.3. The Importance of Plant Proteins in the Context of Global Sustainability

On average, livestock products account for 40% of human protein intake worldwide⁵². The share of protein obtained from animal sources ranges from 18% to 63% of total intake, depending on a country's propensity. Wealthier nations have a significantly larger proportion of animal protein and a higher overall protein supply compared to low-income countries^{52,53}. This statistic is shown in Figure 2.5. The total protein supply is presented in grams per capita per day, where "capita" refers to the individual. In 2022, it ranged from 63 g/capita/day in low-income countries to 110 g/capita/day in high-income countries. In poorer nations, the total protein supply is lower due to limited food availability⁵³. It is important to note that these figures represent supply data. On average, actual consumption in wealthier regions is about 30% lower, as food waste is not covered in the supply figures⁵⁴.

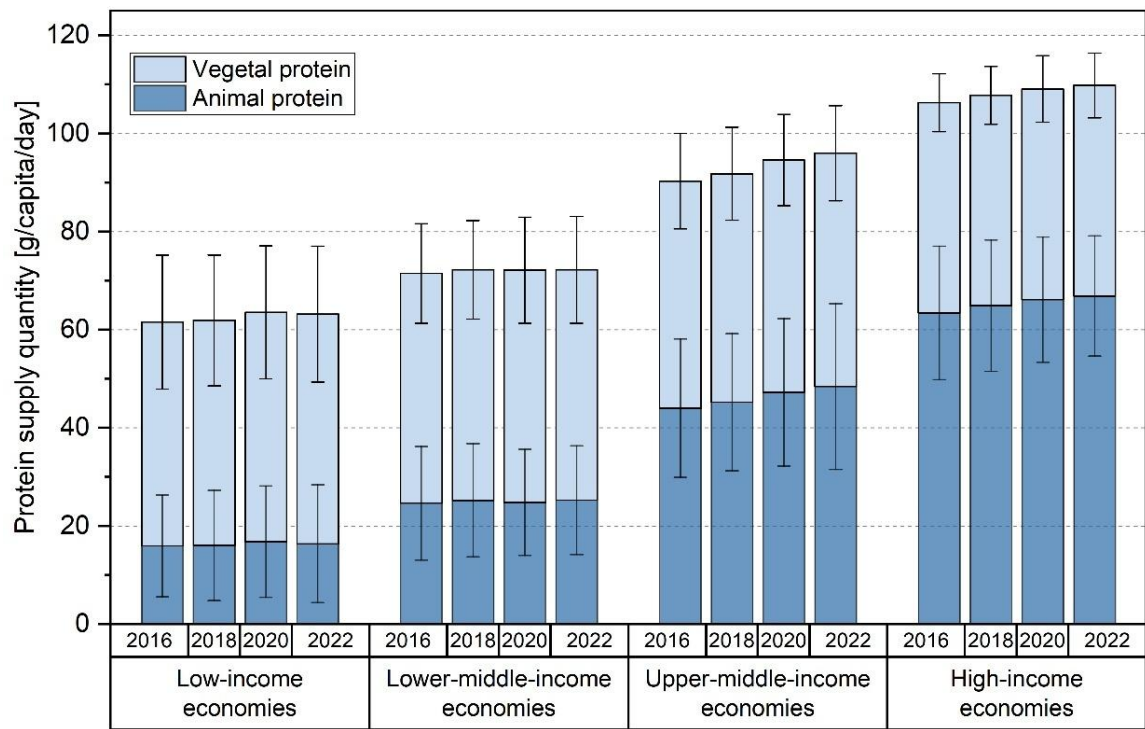


Figure 2.5: Protein supply quantity from vegetal and animal sources, given in g/capita/day. The data are categorized by the economic income into low-income, lower-middle-income, upper-middle-income, and high-income economies. The raw data are obtained from the Food and Agriculture Organization of the United Nations (FAO), database FAOSTAT Food Balances (2010-)⁵⁵. The countries whose data were averaged for the respective income economy were selected according to the categorization provided by The World Bank⁵⁶. Both data sources are licensed under CC BY 4.0. The diagram was inspired by Burlingame *et al.*⁵³.

Wealthier nations consume more animal protein for several reasons: first, the consumption of animal products is both historically rooted and culturally established. Second, the desire for a 'modern' diet increases as prosperity rises; meat is often seen as a status symbol, and alongside urbanization, diets shift from locally grown vegetables to more processed foods. This trend is particularly evident in China and Southeast Asia. Simultaneously, advancements in breeding and feeding technology in the livestock sector have led to significant increases in productivity. The resulting price declines since the 1950s have encouraged greater consumption of animal products, especially in Europe and the US⁵⁷. When considering these development factors alongside global population growth, the demand for livestock products, especially meat, is expected to increase by 50-70% by 2050^{58,59}. But what is the issue with livestock protein? 44% of the world's habitable land area is used for agriculture. About 80% of this is used for animal farming, including grazing land and cropland for feed⁶⁰. Producing protein (and calories in general) from animals is very inefficient: generating one gram of protein from beef or lamb requires nearly 50 to 100 times more land than producing the same amount from peas or soy⁶¹ (see Figure 2.6). In addition, livestock farming accounts for a significant share of global water use (around 41% of the total agricultural water used)⁶² and contributes the largest share, up to 20%, of anthropogenic greenhouse gas emissions⁵⁹. It is also a driver of the nitrogen and phosphorus cycle disruption and, therefore, a driver of eutrophication⁶³. All these factors mentioned above are key contributors to the loss of biodiversity, and the livestock sector has a disproportionately large impact on that⁵⁹.

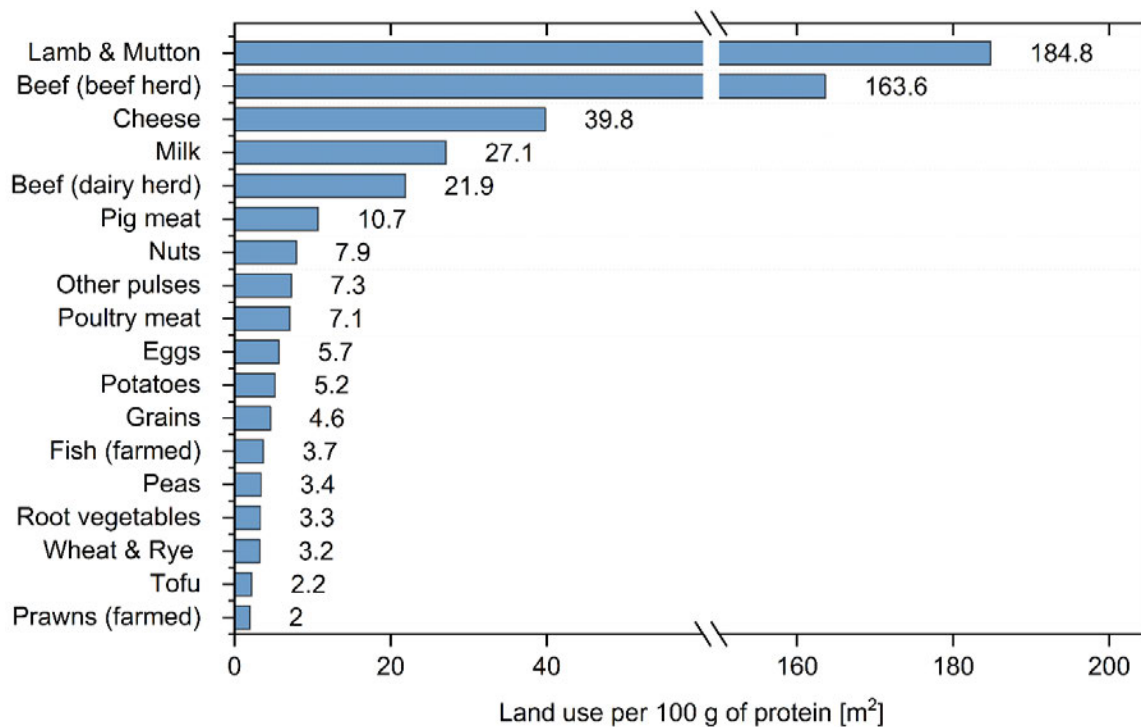


Figure 2.6: Land use per 100 grams of protein. Graphic created based on Our World in Data⁶¹ (CC BY 4.0) and their data source Poore and Nemecek⁶⁴.

The entire food system is one of the most significant drivers of environmental change⁵⁴. Transforming this system is crucial to achieving nations' environmental targets, such as the Sustainable Development Goals (SDGs, 17 goals to achieve peace and prosperity for the planet) established by the UN in 2015⁶⁵ and the Paris Agreement on climate change in 2015 (limit global warming to under 2 °C)^{54,66}. Not only the environment's health, but also human health is directly affected. Inappropriate nutrition, including malnutrition, undernutrition, and overnutrition, is the leading cause of disease worldwide. As the planet reaches its capacity with the current food system and population growth, meeting the increased demand for food resources and distributing them becomes even more challenging in subsequent years⁵⁴. Considering the growing awareness of health, climate change, environmental protection, animal welfare, and the need for food security, the so-called *protein transition* is increasingly part of scientific, political, and societal discussions. This term describes the rebalancing of the protein intake from animal and alternative sources. *Alternative protein sources* include proteins from plants, insects, algae, and fungi, rather than the 'classic' animal protein sources of meat, fish, eggs, and dairy products⁶⁷.

A primarily plant-based diet is the most promising solution based on current research due to its greater sustainability, good consumer acceptance, low cost, high availability, and good nutritional value^{54,67–69}. On average, plant protein sources have comparatively lower protein content, lower digestibility, and an incomplete amino acid profile (lacking essential amino acids) compared to livestock protein; therefore, they are of lower quality than animal-based protein^{52,69–71}. Yet, many studies have

concluded that plant-based proteins provide an adequate and complete protein supply when combined with complementary plant protein sources^{54,72–74}. Meeting the global challenge of the food system transition can only be achieved through a combination of many essential factors. Some have already been mentioned, but different approaches, such as reducing food waste and improving redistribution (whether animal or plant-based), are equally important. Further, the solutions can only be successfully implemented if consumers, governments, and industry work together⁵⁴.

In addition to their central role in nutrition, proteins are multifunctional molecules with an amphiphilic structure. This characteristic allows for solubility in both aqueous and oily systems, as well as gelling, emulsifying, foaming, and structure-forming capabilities. These functional properties can be used to create sustainable films, capsules, surfactants, and carriers for bioactive compounds. This field has predominantly explored animal-derived proteins, which should be replaced with plant-based proteins for the same reasons discussed above, and to be independent of religious or ethical views. Another intensively studied area is the production of meat analogues from plant proteins due to their texturizing properties. Most of the time, those properties are not directly accessible, and the proteins must be (re)functionalized^{68,75–80}. The next chapter provides an overview of various plant protein sources. The subsequent chapters discuss extraction and modification methods.

2.2.4. Sources of Plant Proteins

2.2.4.1. General Overview

Every plant contains proteins. However, extracting them on an industrial scale is not feasible or profitable for every plant, as the protein content, cost, and availability of raw material vary^{81,82}. Different plant protein sources are presented in Figure 2.7. The well-established and large-scale extractable sources are described in more detail.

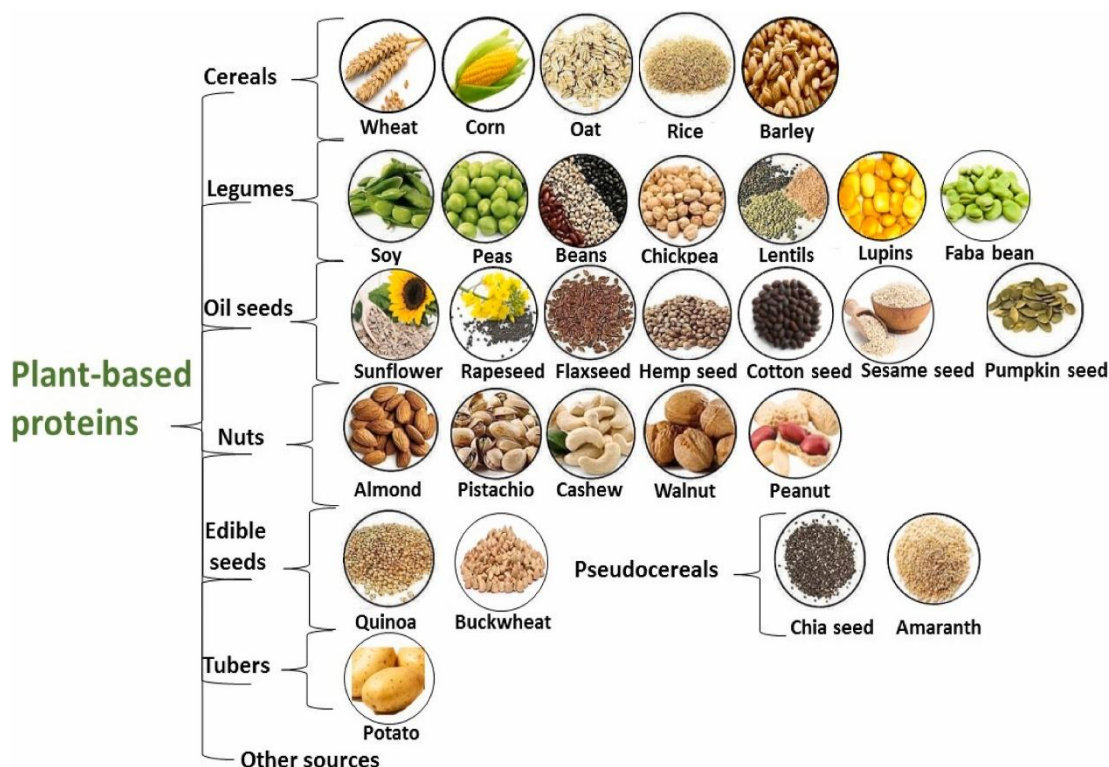


Figure 2.7: Overview of different plant protein sources. The figure is reprinted with CC BY 4.0 license from Nasrabadi *et al.*⁸³.

Cereals are a staple food grown in many regions worldwide, accounting for over 60% of the global food consumption. Proteins are extracted from the grains. Cereals are rich in sulfur-containing amino acids but low in the essential amino acids lysine, threonine, and tryptophan. To achieve a complete amino acid profile, they can be combined with pulse proteins, which are rich in lysine but low in sulfur-containing amino acids. The overall protein content is generally low, with around 8% of the dry mass being protein. Oats are an exception, containing 12-24% protein^{84,85}.

After cereals, pulses are the second most important source of plant protein globally. Proteins are extracted from edible seeds, which have a high protein content of around 20 g per 100 g dry mass, depending on the sort and genotype^{81,86}. A more detailed portrait of two pulse types is provided in the next chapter.

Oilseeds offer a sustainable option, as proteins are extracted from the press cake, a by-product of oil extraction. They generally possess a well-balanced amino acid profile, although most oilseeds are low in sulfur-containing amino acids. The total protein content is relatively high, ranging from 35 to 60 g per 100 g of dry mass, depending on the sort, genotype, and geographical location^{85,87}.

In the nutritional context, plant proteins usually refer to globular storage proteins. Storage proteins provide energy to the plant and are found in the cell's vacuole or cytosol. They comprise the largest proportion of proteins, along with smaller amounts of structural proteins (part of the cell wall) and enzymes (catalysts and drivers of the cell's metabolism). The latter lack nutritional value but are of technological interest. Since nutrition is the primary driver in the protein transition, storage proteins are the main target of extraction⁸⁷⁻⁹⁰.

2.2.4.2. Portrait of Selected Plant Protein Sources

Pisum sativum L., commonly known as pea, and *Cicer arietinum* L., commonly known as chickpea, belong to the family of pulses, lat. *Fabaceae*⁹¹. Figure 2.8 depicts botanical drawings of the plants, as well as their fruits and seeds (pea on the left side, chickpea in the middle).

***Pisum sativum* L.** covers various pea sorts, including garden and field peas. The characteristics of different field pea varieties are very similar to each other but differ from garden peas. Pea is cultivated for human consumption and livestock feed in over 90 countries, with Canada as the largest producer. According to the Food and Agriculture Organization of the United Nations (FAO), the pea production totaled 35.2 million tons in 2023. In the following, the term 'pea' refers to field peas, as they are more relevant for industrial pea protein extraction and account for 13.8 million tons of total pea production. On average, the dry mass of the crop consists of 20-25% protein, 1.5-2.0% fat, 24-49% starch, and 10-20% fiber. They are good sources of B vitamins and potassium^{75,77,91,92}. Plants, in general, also contain inedible and even toxic compounds, called anti-nutrients. These include phytates, saponins, phenolic compounds, tannins, oxalates, and enzyme inhibitors. They do not participate in the plant metabolism but serve various biological functions, such as acting as allelochemicals (substances that influence germination or growth) or phytoalexins (substances produced as defense). In pulses, antinutrients are primarily located in the cotyledons and seed coats, so they are usually removed before consumption. Remaining antinutrients can often be reduced through cooking^{79,93,94}. The composition of peas, especially the protein content, varies depending on cultivar, environmental conditions in cultivation, and breeding progress^{95,96}. Pea protein comprises 55-65% globulins, 18-25% albumins, 4-5% prolamins, and 3-4% glutelins⁹¹. Globulins are the storage proteins in pea and are sometimes reported in even higher proportions⁹⁵. The globulin fraction in pea consists of 11S legumin, 7S vicilin, and convicilin. The additives 11S and 7S refer to the sedimentation coefficients with 'S' as the Svedberg unit. 11S Legumin is a hexameric protein with a molecular weight of 320-380 kDa, composed of six non-covalently linked

subunits of equal weight. These subunits comprise a basic and an acidic polypeptide chain linked by a disulfide bond⁹⁷. The pI of 11S Legumin ranges from 4.8 to 4.9. At pH 7-9 and low ionic strength, the protein maintains its quaternary structure. Shifting the pH outside this area or increasing the ionic strength above 0.05 M causes it to dissociate into its subunits⁹⁸. The amino acid sequence contains high levels of glutamic acid, alanine, valine, and leucine, but low amounts of cysteine and methionine. 7S Vicilin is a trimeric protein with a molecular weight of around 150-180 kDa. Its subunit structure is less straightforward, as it varies due to post-translational processing. The three subunits have a molecular weight of around 50 kDa, are glycosylated, and are linked via hydrophobic interactions^{22,97}. It lacks sulfur-containing amino acids and thus, cannot form covalent disulfide-bridges⁹¹. It is also low in tryptophan but contains high levels of arginine, lysine, aspartic acid, and glutamic acid²². O’Kane *et al.* obtained dissociated lower molecular weight subunits with pH 7.6 and varying salt concentrations between 0.075-0.5 M NaCl. They isolated all globulin fractions at pH = pI = 4.8⁹⁹. Convicilin has a molecular mass of 71 kDa and can form a trimeric quaternary structure. It is often isolated with a highly charged N-terminal extension, increasing the molecular weight. The pI was found to be 5.6-5.8^{22,99,100}. Convicilin shares about 80% of its amino acid sequence with vicilin subunits but comprises sulfur-containing amino acids⁹¹. Due to its close relation to vicilin, there is a debate in the literature whether convicilin should be classified as a separate globulin or a subunit of vicilin^{99–101}. The ratio of legumin to vicilin varies depending on the cultivar and genotype; convicilin accounts for the smallest proportion^{91,95,97}. Albumins extracted from pea mainly consist of enzymes, enzyme inhibitors, and lectins. They have molecular weights around 5-80 kDa. Prolamins and glutelins are also storage proteins. Prolamins typically have high proline and glutamine content, while glutelins contain high levels of methionine and cysteine and are part of gluten. Both fractions are less well-characterized^{77,101}. Protein extraction from peas is appealing because the raw material is widely available and inexpensive. Additionally, pea protein is highly digestible and has good nutritional quality with a balanced amino acid profile (except for its low sulfur content). In many respects, pea is an attractive alternative to soy, the most researched plant protein, as it is less allergenic and clean-labelled, meaning it is not genetically modified organism (GMO)-derived^{22,91}. On an industrial scale, it is extracted via alkaline extraction, followed by isoelectric precipitation and salt extraction^{22,75,77}. Extraction methods are explained and discussed in the next chapter.

***Cicer arietinum* L.** describes two main chickpea varieties: desi and kabuli chickpeas. They differ in their growing regions and appearance: desi chickpeas have small, brown seeds with higher fiber content, while kabuli chickpeas have larger, cream-colored seeds with higher fat content. The desi variety is primarily cultivated in semiarid climates, such as in India and East Africa. The kabuli variety can grow in more moderate climates, such as the Mediterranean region, the Middle East, and North America^{102,103}. The protein contents of the two variants are reported at different levels^{102–104}, and many

publications do not specify the variety but evaluate chickpea in general. Due to the lack of clearly separated data, the following paragraph addresses this similarly. *Cicer arietinum* L. is the third most widely cultivated pulse (after beans and peas) for human nutrition, with a production volume of 16.5 million tons in 2023, according to the FAO. India and the Middle East are the leading producers^{92,105}. On average, the dry mass of chickpea consists of 16-27% protein, 3-6% fat, and 52-71% carbohydrates. The carbohydrates are mainly starch (58-79%), followed by fiber (12-21%), and sugar (6-7%). The carbohydrate level is higher in chickpeas than in the average of pulses. They are good sources of carotenoids and tocopherols^{102,104,106}. Chickpea protein comprises 53-60% globulins, 18-24% glutelins, 8-12% albumins, and 3-7% prolamins, with varying amounts of albumins and globulins, depending on the cultivar and extraction method^{106,107}. Like in peas, globulins are the primary storage proteins in chickpeas, including 11S legumin and 7S vicilin. In contrast to pea, 11S legumin generally constitutes a larger proportion than 7S vicilin^{98,102,106,107}. The structures of legumin and vicilin in pea and chickpea are very similar, with differences in peptide sequences of subunits. The albumin fraction of chickpea also consists of low-molecular-weight enzymes and metabolic proteins and has high nutritional value, as it is sulfur-rich^{98,102}. However, the above-average glutelin content in chickpeas should be emphasized. With their higher amounts of cysteine and methionine, they make chickpeas nutritionally more valuable in terms of a balanced amino acid profile^{101,102}. Protein extraction from chickpeas is appealing because the raw material is readily available, inexpensive, and poorly allergenic. Additionally, chickpea protein is highly bioavailable, and its essential amino acid profile is well-balanced¹⁰⁸. On an industrial scale, it is extracted via alkaline extraction, followed by isoelectric precipitation and salt extraction¹⁰⁵.



Figure 2.8: From left to right: Botanical drawings of *Pisum sativum* L.¹⁰⁹ (graphic of public domain), *Cicer arietinum* L.¹¹⁰ (photo reprinted with permission of Antiquariat Steffen Völkel), and *Solanum tuberosum*¹¹¹ (graphic reprinted with CC BY 3.0 license).

Solanum tuberosum L., commonly known as potato, belongs to the family of nightshade plants, lat. *Solanaceae*^{112,113}. Potato is a global staple food. According to the FAO, 383 million tons of potatoes were produced in 2023, with China as the leading producer^{92,114}. The dry mass of a potato consists of approximately 60-80% starch and 1-2 % protein. The remaining fractions include fibers, minerals, and fats. The tubers are also good sources of vitamins B₃, B₆, and C, along with minerals magnesium, potassium, and iron¹¹⁵⁻¹¹⁷. Potato protein is extracted from the potato fruit juice (PFJ), a low-value by-product of the starch production. PFJ contains about 5% (w/w) dry substance, which includes approximately 35% sugars, 35% proteins, 20% minerals, as well as organic acids, and phenolic compounds^{116,118}. Potato protein mainly comprises albumins and globulins, and to a lesser extent prolamins and glutelins (9% and 2-4%, respectively)¹¹⁹. The main protein fractions are patatins, which account for 30-40% of total protein, and protease inhibitors, contributing 40-50% of total protein, along with higher-molecular-weight enzymes. The composition varies depending on the variety and growing period¹¹⁴⁻¹¹⁷. Patatins, also known as tuberins, are dimeric globulin glycoproteins with a monomeric molecular weight of 40-45 kDa. The monomers are linked via non-covalent hydrophobic interactions, and the structure is present at neutral and alkaline pH and ambient temperature. Lowering the pH below 4.5 or increasing the temperature above 45 °C leads to denaturation and coagulation. Patatins are the main storage proteins in potato, but also serve an enzymatic function as phytoalexins. The pI of patatins ranges between 4.5 and 5.2. They have a good nutritional profile with a high level of the essential amino acid lysine, and their nutritional value is comparable to that of hen egg white^{115,116,119-121}. Protease inhibitors, also known as tuberinins, are a diverse group of monomeric, dimeric, and pentameric proteins that are categorized into subgroups based on their protease-inhibition activity. The molecular weights range from 4.3 to 40 kDa. The pIs range from 5.7 to >9.0. The subunits are linked via disulfide bridges, making their structure more stable than that of patatins. No significant changes occur in their structure across pH 3.0 to 7.5^{115,116}. Although rich in methionine, they are considered anti-nutritional because they hinder protein absorption and digestion. Protein extraction from potatoes is appealing because it upcycles a low-value byproduct into a low-cost and high-availability product. Additionally, potato protein is a less allergenic source of lysine (compared to wheat)^{115,117,119,122}.

2.2.5. From Plant to Functional Protein: Extraction and Modification of Plant Proteins

2.2.5.1. Extraction of Plant Proteins

The target proteins are primarily located in the cell's vacuole and cytosol, protected by a strong cell wall⁸⁸. They also coexist with other undesirable substances, such as carbohydrates, lipids, and secondary metabolites¹²³. Plant protein extraction involves four steps: breaking the cell wall, solubilization of the proteins, precipitation of the proteins, and concentration of the fractions¹²⁴. While cereals are the primary source of plant proteins in nutrition, seeds, followed by pulses, are the major target for protein extraction¹²⁴. Various methods exist for extracting and isolating these proteins from the raw material. Generally, a distinction is made between conventional and non-conventional extraction methods, as well as dry and wet techniques^{88,125}.

All **conventional methods** start by removing fats from the raw material using organic solvents like n-hexane or petroleum ether, followed by milling. Then, proteins are extracted using either wet or dry techniques. Wet techniques solubilize the proteins, followed by precipitating them from the solution. Depending on the extraction medium, these methods are classified into aqueous or organic solvent extraction and alkali-based extraction. Precipitation techniques include organic solvent precipitation, salt precipitation, and isoelectric point precipitation^{88,123}. The most commonly used techniques are alkaline extraction followed by isoelectric precipitation, salt extraction followed by dialysis, and micellar precipitation (where proteins solubilized in salt solution are precipitated by reducing the salt content, forming micelle-type structures)¹²⁶.

In alkaline extraction, the pH of an aqueous suspension of plant material is adjusted to pH 8-12 with NaOH or KOH, often combined with heating. This disrupts the cell matrix, ionizes the proteins, and makes them soluble. Insoluble fractions are removed via centrifugation. With isoelectric precipitation, the pH of the protein solution is adjusted to the proteins' pI, decreasing solubility and causing proteins to precipitate. Alkaline extraction followed by isoelectric precipitation is most common due to its simplicity, efficiency, and cost-effectiveness^{88,123,127}. However, it is not very selective, it uses large amounts of potentially harmful solvents, consumes significant energy, and may result in low protein yield due to harsh extraction conditions. Additionally, only proteins with the specific pI are recovered; other proteins with different pIs remain soluble^{19,123}.

Another method is acid-thermal coagulation. It is used industrially to extract potato protein from potato fruit juice (PFJ), a watery by-product of starch processing. The pH of the PFJ is adjusted to 2 to 6, usually with hydrochloric acid, phosphoric acid, or sulfuric acid. Then the temperature is raised to

above 90 °C, causing the proteins to coagulate and precipitate. This process yields high amounts of protein but results in their complete denaturation and is not very selective. For this reason, acid-thermal coagulation is mainly used to produce protein for animal feed^{115,117,119}. The Dutch company Avebe is, to date, the only company that has successfully established a native extraction process on an industrial scale. In their process, the PFJ is adjusted to a pH of 4 to 6.5 and loaded onto a column equipped with a functionalized support carrier. Proteins are absorbed and isoelectrically precipitated at ambient temperature. Elution is performed in aqueous systems at pH < 3 or pH 6 to 9, depending on the protein fraction. The obtained protein solutions are concentrated by membrane ultrafiltration and dried. The resulting proteins are highly functional and versatile in plant-based foods. The product is sold under the brand Solanic^{®128,129}.

Wet extraction methods require subsequent drying to improve protein handling and storage stability. On an industrial scale, spray drying is preferred for its speed and high throughput, although elevated temperatures may lead to protein denaturation. In comparison, lyophilization is commonly used at laboratory scale due to its gentler conditions; however, it is time-consuming, energy-intensive, and expensive. Comparative studies indicate that drying methods have different effects on protein functionality, emphasizing the need for parameter optimization to preserve it⁸¹. Conventional dry methods involve mechanical separation from undesirable cell compounds based on particle size and density differences, using air classification, sieving, and electrostatic separation^{88,123}. The last step describes the separation of charged particles in an electric field¹²⁵.

Current conventional extraction methods prioritize maximum protein recovery, with little regard for maintaining protein functionality⁸⁸. As a result, commercially available plant proteins are heterogeneous protein mixtures containing impurities, such as carbohydrates and fibers, and are often extensively denatured and thus limited in their functional performance^{17,75,81,126}. Currently, higher functionality is associated with lower protein yield¹²⁶. Although awareness of the importance of functionality in determining the applicability of extracted proteins has grown within the scientific community, this understanding has not yet been widely translated into industrial practices¹²⁵. But what are the measurable parameters that define protein functionality? Protein functionality is associated with the intact native protein structure¹³⁰. Tanger *et al.* systematically studied the conventional wet extraction procedures mentioned above on pea. They identified the combination of solubility and thermal properties as the best indicators for functionality. Additionally, they summarized that different extraction and precipitation methods yield different protein compositions. The facts that plant material from different sources varies in protein content and composition, that established extraction processes yield different protein compositions, and that different methods of calculating protein solubility exist, make comparing scientific studies and industrial powders very challenging^{17,126}.

With the current state of plant protein extraction, two research areas have emerged: on one hand, there is a demand for extraction methods that are less environmentally harmful and cause less denaturation, summarized as **non-conventional methods**. Enzyme-assisted extraction is an innovative wet extraction process that employs cell wall-degrading enzymes, such as cellulases and pectinases, to release the target proteins. Proteases then separate the proteins from polysaccharides and hydrolyze high-molecular-weight proteins. This method produces high-quality protein extracts and is more environmentally friendly than conventional wet techniques. However, it remains expensive, energy-consuming, and challenging to scale up. Another approach is reversed micelle extraction, where proteins are extracted into the aqueous core of reversed micelles in organic solvents, thereby protecting them from harsh conditions. Developing mild methods for extracting proteins from the micelle core is currently a focus of research. This method is considered promising because of its potential to recover and reuse the solvents. Nonetheless, it is still limited to laboratory scale. When combined with innovative physical techniques, the protein yield from non-conventional methods could be enhanced, while the amount of solvent required and the denaturing effect of conventional methods could be reduced. Physical techniques aim to disintegrate the cell wall via ultrasound treatment, microwave radiation, or high-pressure-assisted techniques^{88,93,125,131}.

On the other hand, a variety of protein powders are available on the market at affordable prices. Depending on their protein content, they are categorized into protein flours (10-20% protein), concentrates (55-60% protein), and isolates (more than 80% protein)^{17,79}. Upgrading these products by modifying them for various applications is a valuable approach to accessing functional proteins, especially in the industry. To unlock the potential of plant proteins, they must be extracted in a less denaturing, more selective manner or re-functionalized. It should be emphasized that, at the current state, for most plant proteins, it is not possible to decide whether to use a native or a re-functionalized protein, as only very few native protein powders are commercially available.

2.2.5.2. Modification of Plant Proteins

As long as mild extraction techniques have not been scaled to industrial levels, re-functionalization remains the most promising approach to use the techno-functional properties of plant proteins. The key factor in accessing these properties is the aqueous solubility of the extracted proteins at moderate pH. The factors influencing (plant) protein solubility have been discussed in chapter 2.2.1 and summarized in Figure 2.4. Between 2021 and 2025 alone, several comprehensive reviews on the current state of plant protein modification approaches have been published^{17,18,26,79,83,124,132,133}, indicating the relevance of this topic. They all follow a similar categorization, and so does the following chapter.

Nasrabadi *et al.* define protein modification as “the process of altering the molecular structure or a few chemical groups of the protein by special methods for the purpose of their techno-functionality and bioactivity improvement” (⁸³, page 2). The modification methods are categorized into physical, chemical, and biological techniques^{17,18,26,79,83,124,132,133}. A selection of modification techniques is illustrated in Figure 2.9.

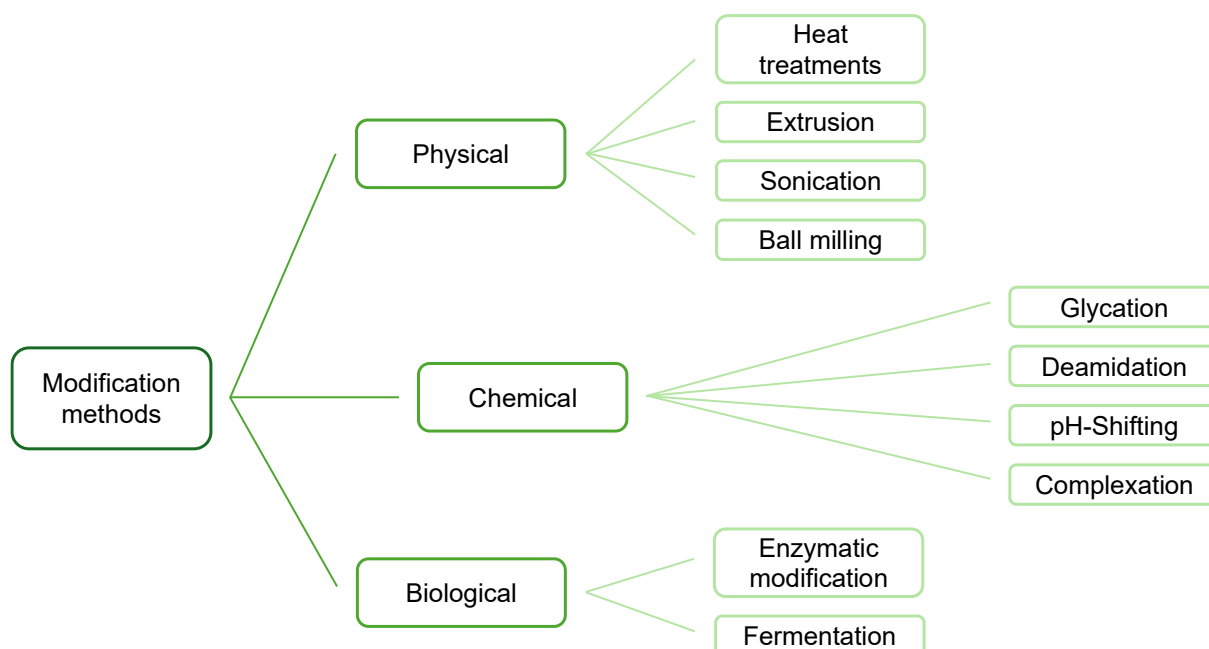


Figure 2.9: Schematic illustration of physical, chemical, and biological plant protein modification methods inspired by Nasrabadi *et al.*⁸³.

Physical modification methods rely on physical forces and energy input; no chemicals are used to change the protein structure. They disrupt non-covalent interactions and induce unfolding. These methods are the most used and can be categorized into heating techniques, sonication, high-pressure treatments, and mechanical downsizing techniques^{83,124}.

Heating occurs through convection, conduction, and radiation. Heat can be applied externally and transported to the target’s surface via convection and conduction. As a result, the target’s surface heats more rapidly and reaches a higher temperature than its interior¹³⁴. Methods that apply external heat are summarized as conventional heating methods. Depending on the temperature and duration of the treatment, heating can promote protein unfolding or irreversible aggregation^{17,83}. Typically, conventional heat treatments are used to reduce the amount of anti-nutritional factors by inactivating enzymes, or to enhance gelling and emulsifying properties by exposing previously buried functional groups. Additionally, raising the temperature accelerates processes and is often combined with other techniques. Infrared heating is an irradiation method that uses infrared radiation to create vibrations of the water molecules, thereby generating heat in the target. Infrared heating consumes less energy and water than conventional methods. In contrast to externally applied heat, heat can also be

generated directly in the target medium. Processes based on this principle are summarized as volumetric heating methods. Ohmic heating uses the medium's electrical conductivity to generate heat by applying an alternating electric current⁸³. Dielectric heating uses a high-frequency electric field produced by electromagnetic waves, such as microwaves and radio waves, to excite polar molecules and ions in the target material, causing them to rotate and move, respectively. Molecular motion generates heat in the medium. Both techniques enable rapid and homogeneous heating and are considered milder than conventional methods, especially in terms of preserving nutritional value. They are established in industry due to their easy and clean handling, high efficiency, and low technical complexity^{83,135}.

Extrusion is a process that combines high temperature, high pressure, and mechanical shear. It can be performed with either high or low moisture content, which affects how the physical forces influence the sample⁴⁵. Extrusion modifies the secondary and tertiary structures of proteins and, consequently, changes their techno-functional properties⁸³. High-moisture extrusion cooking favors hydrogen bond-stabilized products, while low-moisture treatment favors hydrophobic interactions. In both cases, disulfide bridges are formed⁷⁹. The effect of extrusion on protein solubility is reported controversially: depending on the processing parameters, protein aggregates in the raw material can be disrupted into smaller aggregates. This usually enhances protein solubility. On the other hand, the combination of heat, pressure, and shearing can cause cross-linking and aggregation, reducing protein solubility. The latter option is widely used in the food industry to texturize plant proteins into meat substitutes^{83,136}.

Sonication typically uses ultrasound waves to generate cavitation, creating local spots with high temperature, pressure, and shear forces in the sample. Sonication treatments can be categorized by frequency and power into high-frequency-low-power and low-frequency-high-power treatments¹³⁷. External cooling is applied to ensure rapid cooling¹³⁸. Sonication reduces the particle size of protein aggregates by disrupting non-covalent interactions, thereby altering their secondary and tertiary structures. Similar to extrusion, the solubility of treated proteins can either increase or decrease depending on the sonication parameters. This method is mainly used to modify the particle size and the gelling and emulsifying capacities of proteins by exposing previously buried functional groups. Sonication is an energy-efficient, cost-effective, and easy-to-use method, but it is not yet applicable on a large scale^{17,19,83,132}.

Ball milling is a mechanical treatment that reduces particle size of compounds. It combines friction, shear, and collision⁸³.

Chemical modifications are methods that alter protein structure by introducing or removing functional groups. These methods affect not only the secondary and tertiary structures but also the primary structure, and thus the pI of the protein^{17,19,124}. Since many chemical modifications encounter regulatory challenges due to the use of chemicals and the generation of side-products, they are not widely established^{83,124}. Only food-grade modifications are presented in the following.

Glycation is the condensation of a free amino group, such as lysine or the N-terminus of a protein, with the carbonyl group of a reducing sugar via the Maillard reaction. It occurs under controlled heating and can be performed in both wet and dry conditions^{17,83}. Since sugars are mostly already present in extracted protein powders, no additives are required¹⁸. By introducing a sugar moiety to the protein, the pI is altered, and the steric repulsion between protein molecules and their surface hydrophilicity increases. These structural changes positively affect the solubility, emulsifying ability, and thermal stability^{19,83}. As lysine is depleted by glycation, the nutritional value of the protein is changed. Glycation is a clean-label modification method, but it is currently only possible on a laboratory scale^{17,83}.

Deamidation refers to the acidic or basic hydrolysis of the amide side group of asparagine or glutamine into carboxyl groups at elevated temperatures. Consequently, this modification method requires considerable amounts of these specific amino acids in proteins, about 2-6%, to influence their techno-functional properties. This is particularly true for cereals and pulses. By introducing an ionizable side group, solubility and emulsifying properties are increased, and the protein structure is altered. Deamidation is reported to positively affect the taste and reduce the allergenicity of plant proteins^{19,83,139}.

Another method of modification is pH shifting to strongly acidic or basic conditions, followed by adjusting back to a moderate pH. Significantly increasing or decreasing the pH encourages protein unfolding, exposing sulfhydryl groups and hydrophobic side chains. Additionally, ionizable side chains are protonated or deprotonated, increasing the protein's reactivity. As electrostatic repulsion rises, aggregation may be reduced at very alkaline pH. This process enhances solubility and is reported to decrease the proportion of covalent disulfide bridges. pH shifting is usually performed with concentrated HCl and NaOH solutions^{17,19,83,124}. It can also promote alkaline hydrolysis of proteins, leading to dissociation of the primary structure and a reduction in particle size¹⁴⁰.

The complexation of proteins with other proteins, polysaccharides, polyphenols, and surfactants aims to enhance their solubility, emulsifying properties, and stability. It can be customized to meet specific functionality and product requirements. Heteroprotein complexes primarily form through heat and pressure treatment or enzymatic reactions. The interaction forces include electrostatic interactions, which depend on the pI values of the proteins, hydrophobic interactions, and hydrogen bonding. These interactions not only enhance protein functionality but also increase their nutritional value when

combined with proteins that have complementary amino acid profiles. Protein-polysaccharide complexes can form non-covalently via pH-dependent electrostatic interactions or covalently through the Maillard reaction. This combination stabilizes the protein against environmental influences, such as temperature, pH, and ionic strength, while masking off-flavors. Additionally, they are widely explored for their emulsifying capacity^{83,132}. The combination of proteins with phenolic compounds alters the protein structure through covalent and non-covalent binding. Complexation with another functional molecule enhances the protein's thermal stability and antioxidant capacity. Solubility and emulsifying capacity are reported controversially, depending on processing parameters. Since polyphenols themselves are of interest, it may be a symbiotic relationship binding them to the protein as a carrier and protective agent¹²⁴. Proteins can also interact with ionic and non-ionic surfactants, altering the amphiphilic properties of the protein and increasing solubility and emulsifying capacity^{83,132}.

Biological modifications include enzymatic reactions and fermentation. Enzymatic reactions can be categorized into hydrolysis and cross-linking reactions, using proteolytic and oxidative enzymes, respectively. These reactions occur with high specificity depending on the enzyme employed, allowing various functionalities to be enhanced^{17,83}. Hydrolysis refers to the cleavage of peptide bonds in the primary structure, which reduces the molecular weight. The resulting increase in entropy leads to an increase in protein solubility¹⁹. Cross-linking enzymes form covalent bonds between amino acid residues. This process is typically used to strengthen and texturize protein structures and to improve gelling^{79,133}.

Fermentation describes the microbial conversion of organic substances under aerobic or anaerobic conditions¹⁴¹. Starter cultures of bacteria, yeasts, or fungi influence protein solubility through proteolytic activity and the release of secondary metabolites, affecting the environmental conditions in the bioreactor^{19,79}. Besides enhancing protein solubility, fermentation has been shown to improve foaming and nutritional properties, such as reducing off-flavors and allergenicity, and increasing the bioaccessibility and bioavailability of nutraceuticals^{79,83}. Fermentation and enzymatic modifications are considered sustainable due to their low energy consumption, the lack of by-products, and their mild reaction conditions. A drawback of biological modification methods is the high cost of some enzymes and the special care required for microorganisms^{18,123}.

Various combinations of the presented modification techniques exist to enhance their effectiveness^{17,83}. It should be emphasized that changing the protein structure can significantly affect nutritional value, properties beyond intended for techno-functionality, and the toxicity of plant proteins. Therefore, the modification method should always be adapted to the final application of the plant protein. The field of plant proteins is extensive and multifaceted, encompassing a wide range of

protein sources, extraction technologies, modification methods, and applications in both food and non-food sectors. This breadth presents many challenges. The varying composition and performance of different plant protein products can complicate formulation, standardization, and regulatory assessment. Trial and error are followed by understanding and optimization. This diversity offers significant opportunities for tailored functionality, nutritional valorization, and sustainability. In the following practical part, the concepts explained will be applied to three different commercial plant proteins to increase their water solubility.

2.3. Experimental Section

2.3.1. Frame of the Experimental Work

Most solubilization experiments were conducted in cooperation with dsm-firmenich AG (Heerlen, the Netherlands), hereafter referred to as dsm-firmenich. The project aimed to improve the water solubility of commercially available plant protein isolates, making them suitable as microencapsulation agents. dsm-firmenich outlined some requirements and guidelines for the treatment of the proteins:

- The protein isolates should be used as supplied without further purification.
- The protein solutions should be suitable for food products. Therefore, food-approved chemicals should be used to solubilize the protein isolates. Some exceptions were made for comprehension experiments.
- No biological modification methods should be applied due to the regulatory effort surrounding “novel foods” and the generation of overly small protein fragments through enzymatic hydrolysis.
- Kinetic stability was more important than long-term stability.
- The resulting conformation of the protein after solubilization was of little significance.
- A concentration of 2% (w/w) should be used for the solubilization experiments.
- The focus was on evaluating different solubilization strategies. Therefore, screening as many approaches as possible took priority over analyzing the solutions.

The calculation of protein solubility and the sample preparation (chapters 2.3.3.2 and 2.3.3.3, respectively) were provided as an internal report to dsm-firmenich. The results and their discussion were made available in a detailed PowerPoint presentation.

2.3.2. Materials

dsm-firmenich provided protein isolates of pea (PPIp), chickpea (CPIp), and potato (PoPIp). In detail, the obtained products were NUTRALYS®F85F-PEA PROTEIN and TUBERMINE® GP-POTATO PROTEIN from Roquette (Lestrem, France), and chickpea protein isolate G910 from ChickP (Rehovot, Israel). The composition of the commercial products was provided by dsm-firmenich and is presented in Table 2.2. Sodium chloride (NaCl, ≥99.5%), L (+)-ascorbic acid (VitC, ≥99% p.a.) and sodium hydroxide solution (NaOH, ≥99.8%, 1 N) were obtained from Carl Roth (Karlsruhe, Germany). N-methyl-D-glucamine, also known as meglumine, (Meg, ≥99.0%), urea (≥99.5%), hydrochloric acid solution (HCl, Titripur, 1 N), and ammonium sulfate ((NH₄)₂SO₄, ≥99.5%, for analysis EMSURE® ACS, ISO, Reag. Ph Eur) were purchased from Merck KGaA (Darmstadt, Germany). Sodium dodecyl sulfate (SDS, ≥98.5%) was obtained from

Sigma-Aldrich (Darmstadt, Germany). Sodium thiocyanate (NaSCN, $\geq 98.0\%$) and cetyltrimethylammonium bromide (CTAB, $\geq 99\%$) were purchased from Fluka AG (Buchs, Switzerland). MilliQ water was produced in-house at 18 M Ω -cm by a MilliQ water purification system. All chemicals were used without further purification.

Table 2.2: The composition of NUTRALYS®F85F-PEA PROTEIN, G910, and TUBERMINE® GP-POTATO PROTEIN provided by dsm-firmenich. The indices a and b denote the information supplied by dsm-firmenich (a) or the website of the manufacturer (b).

	NUTRALYS®F85F- PEA PROTEIN (PPIp)	G910 (CPIp)	TUBERMINE®GP- POTATO PROTEIN (PoPIp)
Moisture	7.6	6.3	8
Ash	4	2.9	2.8
Total protein	77.8 ^a , 85 ^b	89.1 ^a	78 ^a , 76 ^b
Total fat	8.6	< 0.1	5
Total Carbohydrates	3.4	8.9	0.5

2.3.3. Methods

2.3.3.1. Protein Characterization and Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was performed under reducing conditions on home-cast discontinuous gels (4%-stacking gel, 12%-separating gel). A sample volume of 20 μ L was mixed with 10 μ L of the protein loading dye (for composition, see SI Chapter 2). The mixture was heated to 95 °C for 10 min, and a volume of 10 μ L was loaded onto the gel. A molecular weight standard PageRuler Prestained Protein ladder from ThermoScientific was loaded additionally in a separate lane. The gels were run for 55 min at a constant voltage (190 V). Protein fractions were stained with Coomassie blue.

Differential scanning calorimetry (DSC): The protein isolates were analyzed without pretreatment using the DSC 8000 from PerkinElmer (Waltham, Massachusetts). The measurements were conducted in a nitrogen atmosphere at a flow rate of 20 mL/min and within a temperature range of 20 to 200 °C, with a heating rate of 10 °C/min.

Thermogravimetric analysis (TGA): The protein isolates were analyzed without pretreatment using the TGA-7 from PerkinElmer (Waltham, Massachusetts). The measurements were conducted in a nitrogen atmosphere at a flow rate of 25 mL/min and within a temperature range of 30 to 900 °C, with a heating rate of 10 °C/min.

Infrared spectroscopy (IR): FTIR-ATR measurements of the protein isolates were conducted without pretreatment using a Varian 670 IR spectrometer from Agilent Technologies Inc. (Santa Clara, CA, USA), equipped with a GladiATR probe head from Pike Technologies (Fitchburg, WI, USA). The spectra were recorded in a spectral range of 4000 to 400 cm⁻¹ at a resolution of 2 cm⁻¹. Sixteen scans were taken per sample and averaged.

Dynamic light scattering (DLS): Samples were prepared dust-free and transferred, without filtration, to cylindrical light scattering cells with an outer diameter of 10 mm. DLS experiments were conducted using a CGS-3 goniometer system from ALV (Langen, Germany) equipped with a vertically polarized 22-mW HeNe laser with a wavelength of 632.8 nm. The device was connected to an ALV-7004/FAST Multiple Tau digital correlator. Measurements were performed in a toluene bath at 25 ± 0.1 °C, at a scattering angle of 90° for 300 s. Data were analyzed with the ALV Correlator Software (V.3.0.414) using regularized fitting¹⁴². Particle sizes are presented as intensity-weighted hydrodynamic radii.

Zeta potential (ζ): The samples were diluted with a 1.58 × 10⁻³ M NaCl solution to protein concentrations of 0.32 mg/mL for PPIp, 0.25 mg/mL for CPIp, and 0.22 mg/mL for PoPIp. The NaCl concentration was chosen based on the ionic strength of diluted pH-shifted samples. ζ was measured using a Malvern ZetaSizer Nano ZS from Malvern Instruments GmbH (Kassel, Germany). All samples were analyzed three times, with mean values and standard deviations calculated.

2.3.3.2. Calculation of Protein Solubility

Protein solubility was evaluated as a relative solubility by calculating the nitrogen solubility index (NSI). It is defined as the ratio of soluble nitrogen to total nitrogen and is expressed as a percentage^{19,143}. The soluble nitrogen was determined indirectly as the difference between the total nitrogen in the protein isolate powder and the insoluble nitrogen remaining in the residue after treatment. It was calculated using equation (2.3), combining the gravimetric data of lyophilized and weighed residues with the percentage of nitrogen obtained via catalytic tube combustion with oxygen supply.

$$NSI = \frac{N(isolate) \cdot m(isolate) - N(residue) \cdot m(residue)}{N(isolate) \cdot m(isolate)} \cdot 100 \quad (2.3)$$

with $N(isolate)$ as the nitrogen content of the protein isolate powder in decimal number, $m(isolate)$ as the used mass of protein isolate powder in mg, $N(residue)$ as the nitrogen content of the protein isolate residue in decimal number, and $m(residue)$ as the mass of the protein isolate residue in mg.

The NSI can be converted into a protein mass concentration using equation (2.4). By applying a conversion factor that reflects the statistical proportion of nitrogen in proteins, the nitrogen solubility is translated into a crude protein solubility^{113,144}. Crude protein solubility includes non-proteinogenic nitrogen, such as free amino acids, peptides, and phospholipids¹⁴³.

$$\beta(\text{Protein}) = \frac{N(\text{isolate}) \cdot 6.25 \cdot m(\text{isolate}) \cdot NSI}{V(H_2O)} \quad (2.4)$$

with 6.25 as the nitrogen to crude protein conversion factor following Ebert *et al.*¹¹³ and $V(H_2O)$ as the volume of water in mL

2.3.3.3. Sample Preparation for Different Treatments

The concentration of protein isolates in water or an aqueous additive solution was consistently 2% (w/w). Volume or mass changes in the sample, such as those resulting from pH adjustments, were disregarded. All treatments began by dispersing the protein isolates in MilliQ water or an additive solution and stirring for 30 minutes to achieve homogenization. Deviations from this procedure are indicated in the preparation description. Stirring was always conducted in the dark at room temperature (RT) and 500 rpm, unless stated otherwise. The term “native pH” describes the pH of an aqueous suspension containing 2% (w/w) of the respective protein isolate. Adjusting to native pH means pH 7.0 ± 0.1 for PPIp and CPIp, and pH 5.0 ± 0.1 for PoPIp. Unless otherwise stated, the pH was adjusted using HCl (1 N) and NaOH (1 N). After completing the treatment and storing the samples at 8 °C in the refrigerator overnight (approximately 18 h), all samples were centrifuged for 15 min at $20379 \times g$. The sample residue was separated from the supernatant and lyophilized for approximately 24 hours. The lyophilized residues were weighed, and the nitrogen content was determined through catalytic tube combustion with oxygen supply. The analysis was conducted at the central analytical department at the University of Regensburg, using a vario MICRO Cube elemental analyzer from Elementar Analysensysteme GmbH, Langenselbold, Germany.

2.3.3.3.1. Reference Solubility in Water

The protein isolate suspensions were stirred for two hours and stored overnight at 8 °C.

2.3.3.3.2. Degassing

The suspensions were degassed for approximately five seconds using a water-jet pump. The vials were vented and stored overnight at 8 °C.

2.3.3.3.3. Sonication

Sonication (US) was performed using an ultrasonic homogenizer UP200Ht from Hielscher Ultrasonics GmbH (Germany). The sonication parameters were inspired by O'Sullivan *et al.*¹³⁷ and adapted to the experimental setup. The following parameters remained constant throughout all sonication treatments:

- Titan sonotrode: S26d7 (diameter 7 mm)
- Immersion depth of sonotrode: ~ 5 mm
- Frequency: 26 kHz
- Amplitude: 80%
- Treatment time: 120 s, not pulsed

Sonication was conducted in an ice bath to minimize temperature effects. The treatment was started at a sample temperature of 10 °C and did not exceed 25 °C. After sonication, the samples were stirred for 30 min and stored overnight at 8 °C.

2.3.3.3.4. Heating

The suspensions were placed in water baths at 22, 40, 60, and 80 °C and stirred for 30 min. After cooling to RT under constant stirring, the samples were stored overnight at 8 °C.

2.3.3.3.5. pH Shifting

The pH was adjusted to pH 2.0 ± 0.1 , 4.0 ± 0.1 , 6.0 ± 0.1 , 8.0 ± 0.1 , 10.0 ± 0.1 , and 12.0 ± 0.1 with HCl or NaOH, and the suspensions were stirred for two hours. After readjusting the pH to native pH, the samples were stirred for 30 min and stored overnight at 8 °C. In a separate test series, the basic pH values were adjusted with an aqueous meglumine solution (1 N) instead of NaOH. The rest of the procedure remained unchanged.

2.3.3.3.6. Additive Screening

Various additive classes were tested to evaluate the influence on the protein solubility in water. Concentrations ranged from 5 to 100 mM. Aqueous solutions of urea, sodium thiocyanate (NaSCN), sodium chloride (NaCl), ammonium sulfate ((NH₄)₂SO₄), sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), and L(+)-ascorbic acid (Vitamin C, VitC) in MilliQ water were prepared at specific concentrations. Protein isolate powders were suspended in the aqueous additive solutions. The samples were stirred for two hours and stored overnight at 8 °C.

2.3.3.3.7. Combination of pH Shifting and Heating

pH 12.0 ± 0.1 was adjusted with NaOH (1 N), and the suspensions were stirred for 30 min. The samples were placed in water baths at 22, 40, 60, and 80 °C and stirred for 30 min. After cooling to RT under constant stirring, the pH was readjusted to native pH. The samples were stored overnight at 8 °C.

2.3.3.3.8. Combination of pH Shifting and Sonication

After adjusting the pH of the suspensions to $\text{pH } 2.0 \pm 0.1$ - 12.0 ± 0.1 , sonication (US) was performed. The samples were stirred for two hours, then the pH was readjusted to the native pH. The samples were stirred for another 30 min and stored overnight at 8 °C.

2.3.3.3.9. Combination of Vitamin C and Heating

An aqueous Vitamin C (VitC) solution with a concentration of 0.3 g/mL ($\triangleq 1.7 \text{ M}$) was prepared, and the effect of three different temperatures on protein solubility was examined. Protein powders were suspended in the VitC solution. The samples were stirred at RT or placed in a water bath at 50 °C and 90 °C. The processing times for the samples at RT and 50 °C were two hours, while the 90 °C samples were treated for ten minutes. After cooling to RT under constant stirring, all samples were stored overnight at 8 °C.

2.3.3.3.10. Combination of Vitamin C and Sonication

The influence of VitC and sonication in combination was tested in two different orders.

First US, second VitC: The protein isolates were suspended in MilliQ water and stirred for 30 min. Sonication was performed, followed by adding VitC powder to obtain a final concentration of 0.3 g/mL in the aqueous protein suspension. The samples were stirred for two hours and stored overnight at 8 °C.

First VitC, second US: An aqueous VitC solution with a concentration of 0.3 g/mL was prepared. Protein powders were suspended in the VitC solution and stirred for two hours. Sonication was performed, followed by stirring for 30 min and storage overnight at 8 °C.

2.3.3.3.11. Combination of Vitamin C, Heating, and pH Shifting

First VitC, second pH shifting: An aqueous VitC solution at 0.3 g/mL was prepared. Protein powders were suspended in the VitC solution. The samples were stirred at RT or placed in a water bath at 50 °C and 90 °C. The processing times for samples at RT and 50 °C were two hours, while the 90 °C samples were treated for ten minutes. After cooling down to RT, the pH was adjusted to native pH. The samples were stirred for 30 min and stored overnight at 8 °C.

First pH 12, second VitC: The protein isolates were suspended in MilliQ water and stirred for 30 min. After adjusting to pH 12, the samples were stirred for two hours. VitC powder was added to obtain a final concentration of 0.3 g/mL in the aqueous protein suspension. The samples were stirred for two hours and stored overnight at 8 °C.

2.3.3.3.12. Combination of Vitamin C and SDS

The influence of VitC and SDS in combination was tested in two different orders.

First VitC, second SDS: An aqueous VitC solution at 0.3 g/mL was prepared. Protein powders were suspended in the VitC solution and stirred for two hours. SDS powder was added to obtain final concentrations of 5-100 mM in the aqueous protein suspension. The samples were stirred for two hours and stored overnight at 8 °C.

First SDS, second VitC: Aqueous SDS solutions with defined concentrations of 5-100 mM were prepared. Protein powders were suspended in the SDS solutions and stirred for two hours. VitC powder was added to obtain a final concentration of 0.3 g/mL in the aqueous protein suspension. The samples were stirred for two hours and stored overnight at 8 °C.

2.3.3.3.13. Combination of SDS and Heating

A 50 mM SDS solution was prepared. Protein powders were suspended in the SDS solution and stirred for 30 min. The samples were stirred at RT or placed in a water bath at 40, 60, or 80 °C. The stirring time for all samples was 90 min. After cooling to RT under constant stirring, the samples were stored overnight at 8 °C.

2.3.3.3.14. Combination of SDS and Sonication

Sonication was performed, then SDS powder was added to achieve final concentrations of 5-100 mM in the aqueous protein suspension. The samples were stirred for two hours and stored overnight at 8 °C.

2.3.3.3.15. Combination of SDS and pH Shifting

The pH was adjusted to pH 12 ± 0.1 and the suspensions were stirred for two hours. SDS powder was added to obtain final concentrations of 5-100 mM in the aqueous protein suspension. The samples were stirred for two hours, then the pH was readjusted to the native pH. The samples were stirred for 30 min and stored overnight at 8 °C.

2.3.3.3.16. Combination of SDS, pH Shifting, and Heating

A 50 mM SDS solution was prepared. Protein powders were suspended in the SDS solution and stirred for 30 min. The pH was adjusted to pH 12 ± 0.1 and the suspensions were stirred for 30 min. The samples were stirred at RT or placed in a water bath at 40, 60, or 80 °C. The stirring time for all samples was 90 min. After cooling to RT under constant stirring, the pH was adjusted to the native pH. The samples were stirred for 30 min and stored overnight at 8 °C.

2.4. Results and Discussion

2.4.1. Characterization of Plant Protein Isolates

Since the isolates contain varying levels of protein (76-89%, see Table 2.2), protein solubility is evaluated as the ratio of soluble nitrogen to total nitrogen (NSI), rather than to the total protein isolate (which includes non-nitrogenous components, such as carbohydrates, ash, and fat). Due to differences in the amounts of non-nitrogenous compounds, referencing the total protein isolate would lead to inconsistent comparisons of protein solubility enhancement across treatments. As the protein isolates contain mainly protein, the solubility of non-nitrogenous components is not considered here. Since a relative solubility is determined¹⁹, protein solubility is expressed as a percentage, rather than in mg/mL. The protein solubility in MilliQ water serves as a reference value. The native pH describes the pH of an aqueous protein suspension¹¹³ and serves as a reference point; if the pH was temporarily altered during treatment, it was adjusted back to the protein isolate's native pH in water, enabling comparison of protein solubility with the untreated reference. As the experiments were part of an overview-oriented screening study, all solubility values are single determinations. For this reason, only significant changes in solubility are discussed in the following chapter. The aqueous solubilities of the protein isolates, as well as their native pH values, are shown in Table 2.3.

Table 2.3: The aqueous solubility and the native pH of the protein isolates NUTRALYS®F85F-PEA PROTEIN (PPIp), G910 (CPIp), and TUBERMINE® GP-POTATO PROTEIN (PoPIp). The native pH describes the pH of an aqueous suspension of the protein isolates at 2% (w/w).

Protein Isolate	Aqueous protein solubility	Native pH
PPIp	16%	7.1
CPIp	33%	7.0
PoPIp	5%	4.9

PoPIp exhibits the lowest protein solubility, with only 5% of the total protein content being solubilized. Its native pH is acidic at 4.9. PPIp and CPIp have similar native pH values around 7, but CPIp is twice as soluble as PPIp. The protein solubilities of commercial isolates at neutral or native pH reported in the literature range from 8% to 61% for pea protein isolates^{75,81,113,126,145,146}, and from 5% to 40% for most potato protein isolates^{113,147}. Solanic® products from Avebe (Veendam, The Netherlands) stand out among commercial potato proteins, achieving protein solubilities of 90-100%^{113,148}. Protein solubilities of around 60% are reported for chickpea protein isolates^{101,105}. Generally, proteins extracted at laboratory scale exhibit higher solubility than their commercial equivalents^{22,75,81}. The variation in solubility arises from the lack of standardized methods and conditions for determining protein solubility, as well as the inherent factors of various products (discussed in detail in section 2.2.2).

Manufacturers provide limited information on cultivar, extraction parameters, or proportions of Osborne fractions in the commercial products. Additionally, it was found that solubility varies between different batches of the same product, and the solubility of identical products decreases during storage (see Table SI 2 1). Similar observations are also reported in the literature^{22,75}. Therefore, each protein solubility experiment must be evaluated within its own reference system.

To evaluate the denaturation state of the protein isolates, TGA (A and B) and DSC (C) measurements of the powders, along with reducing SDS-PAGE (D) of the aqueous solutions, were performed (see Figure 2.10). Infrared spectroscopy measurements are provided in Figure SI 2 1 and Table SI 2 2.

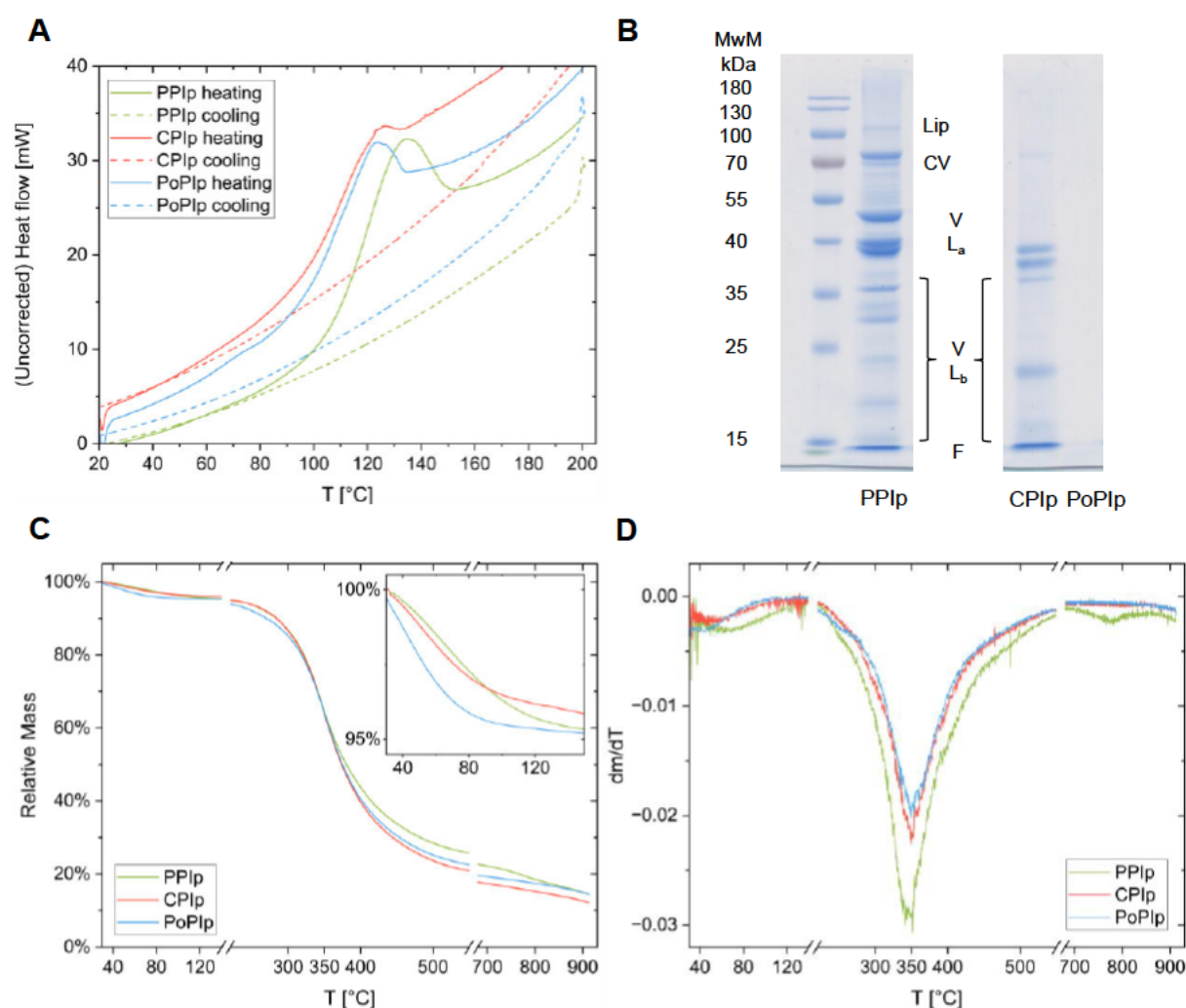


Figure 2.10: DSC (A), reducing SDS-PAGE (B), and TGA (C and D) of PPIp, CPIp, and PoPIp.

The DSC results (A) are similar for all three protein isolates. The thermograms display a single, broad endothermic peak with onset temperatures between 93 and 104 °C and peak maxima between 124 and 134 °C. The peak can be attributed to the vaporization of moisture from the powder. The lack of endothermic peaks between 70 and 90 °C for PPIp and CPIp indicates that these proteins are already denatured^{81,126,146,149–152}. The DSC data of PoPIp show a very flat peak at 72 °C, indicating the presence of native protease inhibitors¹⁵³. The TGA measurements (C) show similar results for all protein isolates,

with a first mass loss between 30 and 110 °C and a second loss starting at 220 °C. The first mass loss is due to moisture loss. The second one is attributed to the degradation of organic matter^{149,154,155}. PoPIp exhibited a smaller slope than PPIp and CPIp at the start of the second mass loss, creating a small shoulder before the main degradation peak in the derivative of the thermogram (**D**). This may be caused by a small native protein fraction that degrades differently than the denatured protein fraction, as suggested by DSC data.

SDS-PAGEs of PPIp and CPIp (**B**) show distinct bands across the size range of the molecular weight standard (15-180 kDa), indicating the presence of various proteins or protein subunits. The bands are roughly classified according to the assignments in reducing SDS-PAGEs reported in the literature. Bands close to the bottom are attributed to albumin or vicilin fragments. Bands from 15 to above 35 kDa are linked to different vicilin 7S polypeptides and the basic polypeptide of the legumin 11S subunit. Bands between 40 and 55 kDa are assigned to vicilin 7S subunits and the acidic polypeptide of the legumin 11S subunit. Convicilin and lipoxygenase are identified in PPIp at around 70 and 100 kDa, respectively^{97,126,146,151,156}. SDS-PAGE of PoPIp shows no clear bands, but a very faint band may appear below 15 kDa. With a solubility of 5% in water, the protein amount in the loading volume is 16.4 µg, which is low but should be sufficient for detection. The absence of bands could either mean the concentration is too low or that the soluble proteins or aggregates are outside the observation range.

The protein isolates exhibit poor water solubility and lack thermal denaturation peaks in DSC measurements, indicating prior denaturation. TGA results confirm residual moisture and nearly uniform decomposition. SDS-PAGE reveals protein subunits or fragments likely present before analysis (not formed during SDS-PAGE pretreatment). Overall, the data suggest that the isolates have reduced functionality due to extensive denaturation.

2.4.2. Modification of Protein Isolates

2.4.2.1. Physical Treatments

The effects of heating, sonication, and degassing on the aqueous solubility of PPIp, CPIp, and PoPIp were studied. The results are presented in Figure 2.11. Particle size and zeta potential (ζ) of the protein isolates were measured after heat treatment (see Figure 2.12). Heating was conducted externally through convection at temperatures of 22, 40, 60, and 80 °C (abbreviated with Tvalue). The solubility of PPIp increases significantly from 16% to 64% with rising temperature during treatment. The particle size and distribution of PPIp remain unchanged when comparing T22 and T80→22, showing two populations with hydrodynamic radii of 9-13 nm and 120-125 nm. ζ also stays constant at -40 mV for both temperatures.

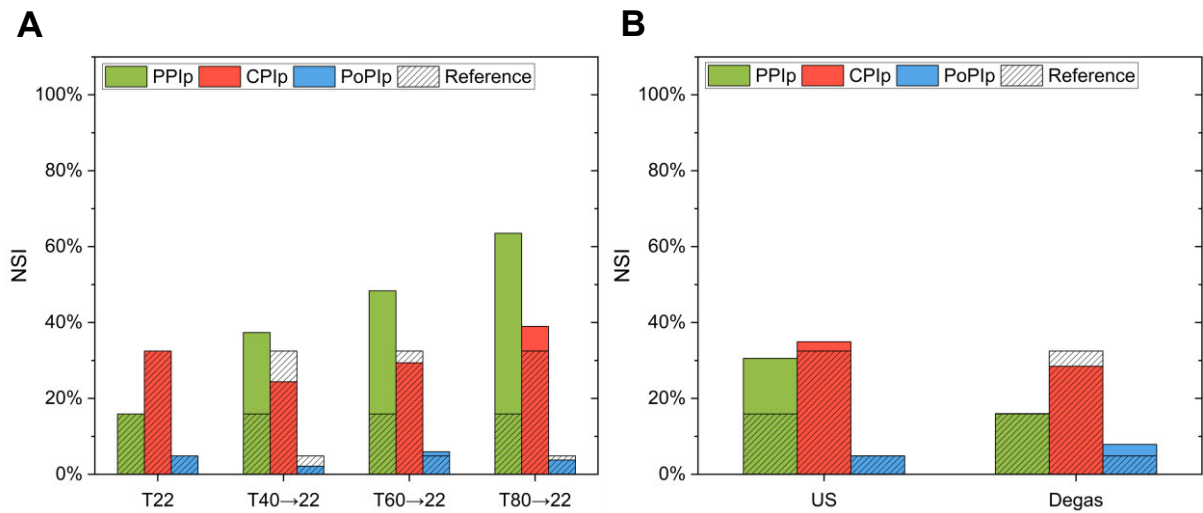


Figure 2.11: Physical treatments for protein modification. Influence of heating to different temperatures (T, **A**), sonication (US, **B** left), and degassing (Degas, **B** right) on the aqueous solubility of PPIp, CPlp, and PoPlp. The reference (hatched lines) marks the reference protein solubility at RT without any treatment. The solubility was evaluated at RT. The pH was not actively set.

The solubility of CPlp drops below the reference at T40→22 and T60→22, then increases at T80→22 to 39%, slightly above the reference. The particle size distribution shows three populations with mean hydrodynamic radii of 3, 12, and 87 nm at T22. Heating to 80 °C reduces the particle size distribution to a single broad peak with a mean hydrodynamic radius of 60 nm and an upstream shoulder. ζ remains steady at -30 mV for both temperatures. The solubility of PoPlp decreases by half at T40→22 and remains nearly unchanged at other temperatures. The particle size distribution stays monomodal at both temperatures, but the peak broadens with heating, shifting the peak maximum from 79 nm at T22 to 75 nm at T80→22. ζ shifts significantly from -20 mV at T22 to -11 mV at T80→22.

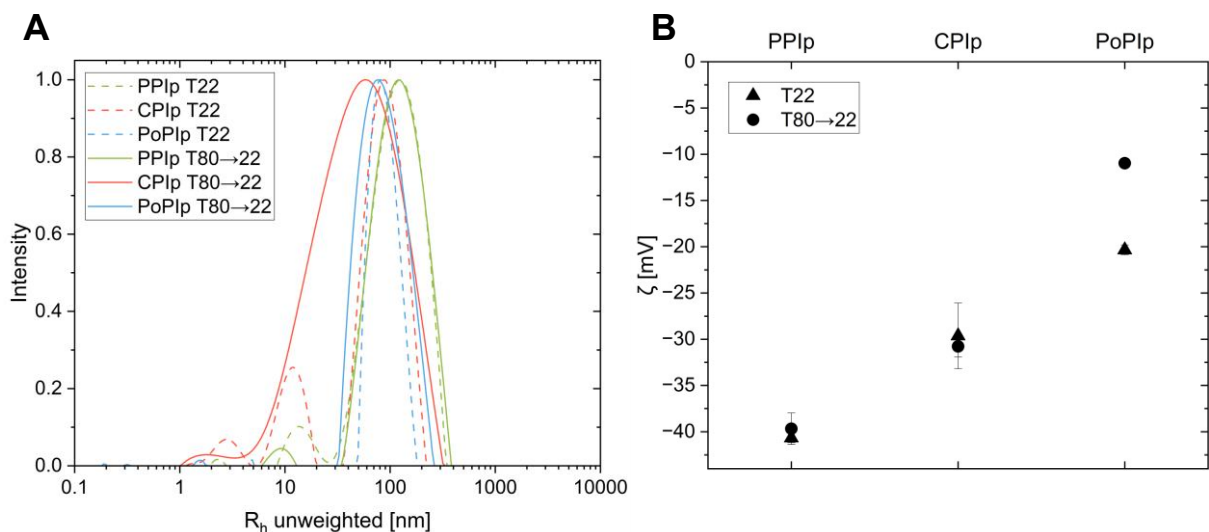


Figure 2.12: Particle size distributions (**A**) and zeta potentials (ζ , **B**) of PPIp, CPlp, and PoPlp at T22 and T80→22.

The DLS results indicate the presence of large aggregates. These are not visible on SDS-PAGE because they may only be partially solubilized in the untreated reference sample and are too large to migrate into the gel. When analyzing DLS data, it should be noted that the intensity-weighted representation of particle sizes overestimates larger aggregates (see section 1.2.3). Therefore, no statement can be made on the ratio of large aggregates to smaller particles.

Heating is typically associated with protein denaturation. Treating already denatured proteins with heat enhances the system's entropy¹⁹ and can lead to the disassembly of protein aggregates by breaking non-covalent bonds. This process may either reduce the size of larger protein aggregates or promote unfolding and elongation of polypeptides, thereby increasing their reactivity towards the formation of new aggregates^{146,157}. Depending on the mechanism and environmental conditions, aggregation is not always associated with protein insolubility, but the formation of soluble aggregates is also possible¹⁵⁸. In both cases, previously buried functional groups and interaction sites are exposed^{146,157}. Therefore, heating is often used to improve emulsifying and gelling properties^{91,159,160}. Messin *et al.* and Chao *et al.* studied the effect of heating on pea globulins and pea protein isolate, respectively. Both confirmed the exposure of hydrophobic groups and the formation of larger soluble aggregates due to random aggregation of subunits. Messin *et al.* also observed a slight increase in free sulfhydryl groups, and Chen *et al.* reported improved emulsification ability for the thermally treated protein isolate compared to the untreated one^{151,161}.

Several studies have investigated how heating impacts globular potato proteins. It was found that potato proteins denature at low ionic strength and neutral pH between 50 °C and 75 °C. Increasing ionic strength raises the denaturation temperature¹⁵³. Heat denaturation occurs in two stages: first, unfolding, followed by increased reactivity and the formation of larger aggregates¹⁴⁸. Heating potato proteins and their isolates is mainly done to improve gelling and foaming properties by unfolding and forming network structures^{114,117}.

In the current case, the increase in PPIp solubility cannot be linked to changes in particle size or ζ . Therefore, it is proposed that reorganizing protein aggregates, considering the shifts in the distribution of small particles, results in improved protein solubility. ζ of CPIp remains unchanged; however, the particle size distribution indicates a random aggregation process, leading to a less-defined size population, which does not significantly affect solubility.

Heating of PoPIp does not alter the particle distribution, but ζ . It is suggested that the current aggregates are covalently cross-linked and therefore too strong to break down by heating. However, a reduction in surface charge explains the decreased solubility upon heating. The pI of PoPIp appears to be lower than that of the native compounds, since ζ at pH 5 is clearly negative and not near zero, which would be expected close to the pI.

Sonication increases PPIp solubility by a factor of 2, while the solubilities of CPIp and PoPIp remain unchanged. Sonication has been reported to have a controversial effect on protein isolate solubility; it can increase solubility by disrupting larger aggregates^{83,108,156}, but may also decrease solubility when applied with excessive energy, promoting aggregation^{19,83}. The sonication parameters (see section 2.3.3.3.3) in this study were selected based on O'Sullivan *et al.*, who reported a solubility-enhancing effect on various protein isolates due to particle size reduction¹³⁷. Heat-induced functionality loss during sonication could be excluded due to the external cooling of the samples. The energy applied appears sufficient to alter a proportion of PPIp aggregates.

Degassing increases the solubility of PoPIp by a factor of 1.6 and slightly decreases CPIp's solubility. It does not affect the solubility of PPIp. The idea was that small, hydrophobic air bubbles in protein suspensions could cause low protein solubility¹⁶². Since degassing is the only physical treatment that impacts PoPIp's solubility, it can be concluded that the presence of hydrophobic spots influences it more than the presence of large aggregates.

2.4.2.2. Chemical Treatments

Albumins and globulins are the most significant proteins in the analyzed protein isolates. They have been classified by Thomas B. Osborne as soluble in water and diluted salt solutions, respectively²¹. As discussed earlier, the aqueous solubility of these protein isolates is low compared to the proportion of albumins reported.

The influence of different salts on protein solubility is shown in Figure 2.13. The addition of NaCl (**A**) does not significantly affect the protein solubility. The influence of NaSCN (**B**) varies: it increases PoPIp's solubility at 5, 10, and 20 mM but decreases it at 50 and 100 mM. NaSCN does not significantly affect the solubility of CPIp. PPIp's solubility decreases as NaSCN concentration rises. The addition of ammonium sulfate (**C**) has a consistent effect across all isolates; protein solubility remains unaffected at lower salt concentrations but drops significantly at higher concentrations. PoPIp's solubility at 100 mM cannot be evaluated due to the co-precipitation of PoPIp and ammonium sulfate. The pH values of all samples are summarized in Table SI 2.3. The addition of the salts does not impact PoPIp's pH and slightly shifts the pH of PPIp and CPIp towards more acidic values.

The influence of salts can be discussed from two perspectives: ionic strength and specific ion effects. The mechanisms underlying these aspects have been detailed in detail in section 2.2.2.4. Increasing ionic strength by adding NaCl does not significantly change protein solubility, suggesting that globulins lack functionality. SCN^- is a strong salting-in anion, while $(\text{NH}_4)_2\text{SO}_4$ is commonly used to precipitate proteins¹⁶³. Despite their different molecular properties, both ions influence the protein solubility similarly. This indicates that ion-specific effects are not significant and that the studied proteins

contain, if at all, an insignificant amount of native protein. Additionally, the change in pH in PPIp and CPIp suspensions upon adding NaSCN and $(\text{NH}_4)_2\text{SO}_4$ could also reduce solubility. These complementary experiments support the findings from the initial characterization studies.

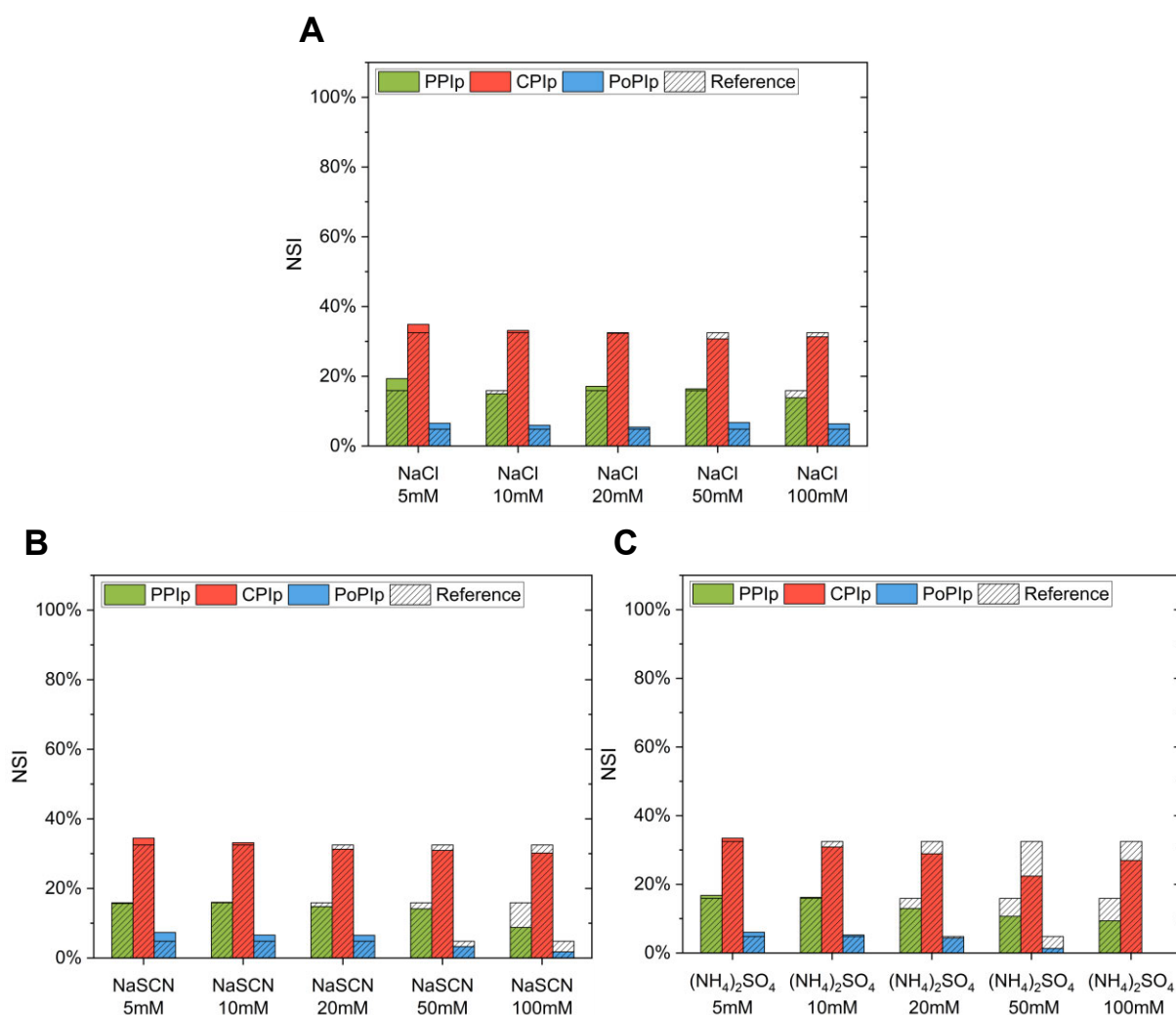


Figure 2.13: Chemical treatment for protein modification. Influence of salts sodium chloride (NaCl, **A**), sodium thiocyanate (NaSCN, **B**), and ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$, **C**) on the aqueous solubility of PPIp, CPIp, and PoPIp. The reference (hatched lines) marks the aqueous solubility at RT without any treatment. The solubility was evaluated at RT, and the respective pH values are summarized in Table SI 2.3.

Shifting the pH affects covalent and non-covalent interactions in protein aggregates^{17,19,83,124,140}. The solubility of PPIp, CPIp, and PoPIp after pH shifting towards strongly acidic or basic levels, followed by readjusting to the native pH, is presented in Figure 2.14. pH shifting with NaOH/HCl towards acidic and basic pH and back to the native pH does not significantly affect the solubility of PoPIp. Switching the base from sodium hydroxide to meglumine increases its solubility by a factor of 1.7-1.8 at pH 10 and 8, respectively. pH 12 could not be adjusted due to the lower pH of the meglumine solution. For PPIp and CPIp, a U-shaped solubility pattern is observed, with minimum solubility at pH 4→n and pH 6→n and increasing solubility for both highly acidic and increasingly basic pH values. pH 12→n results in the most significant increase in solubility, with 40% and 52% for PPIp and CPIp, respectively.

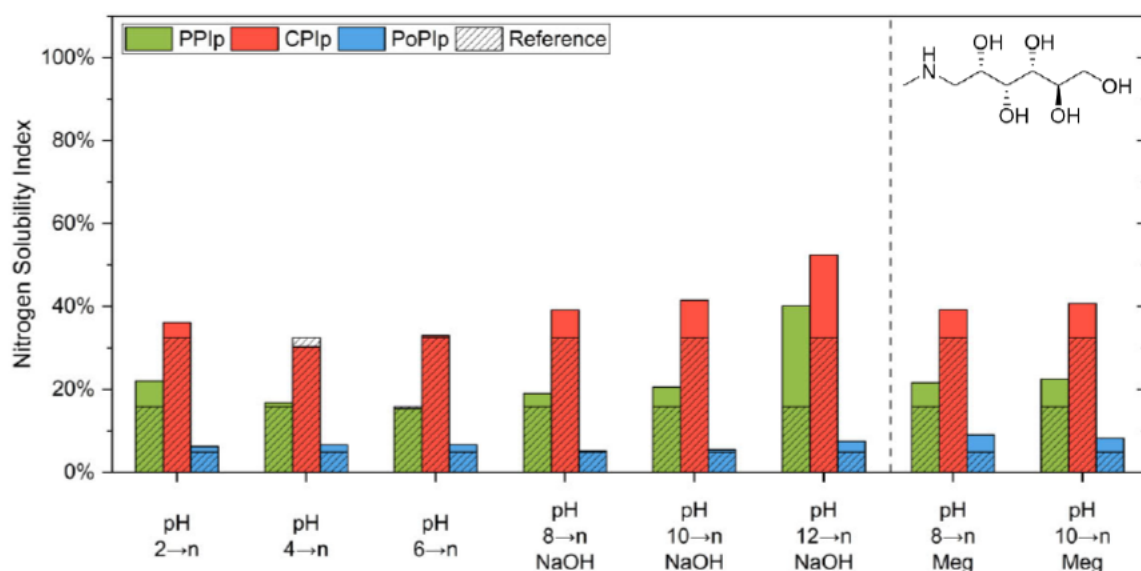


Figure 2.14: Chemical treatment for protein modification. Influence of pH shifting on the aqueous solubility of PPIp, CPIp, and PoPIp. The alkaline pH values were adjusted with NaOH and meglumine solution (Meg, molecular structure in the diagram). pH value $\leftrightarrow n$ describes the shift from native pH (n) to the target pH (value) and back to native pH. The reference (hatched lines) marks the aqueous solubility at RT without any treatment. The solubility was evaluated at RT and native pH.

Switching the base to meglumine does not affect PPIp solubility but slightly increases CPIp solubility at pH 8 $\rightarrow$ n and pH 10 $\rightarrow$ n compared to NaOH. The particle size distribution and ζ of the protein isolates were measured for pH12 $\rightarrow$ n and are presented in Figure 2.15. The particle size distribution of PPIp and PoPIp shifts toward smaller mean hydrodynamic radii of 99 and 57 nm, respectively, and moves toward larger mean hydrodynamic radii for CPIp (138 nm). The particle size distribution of PoPIp broadens significantly. ζ shifts to -34 mV for PPIp and to -10 mV for PoPIp, respectively. ζ of CPIp remains unchanged.

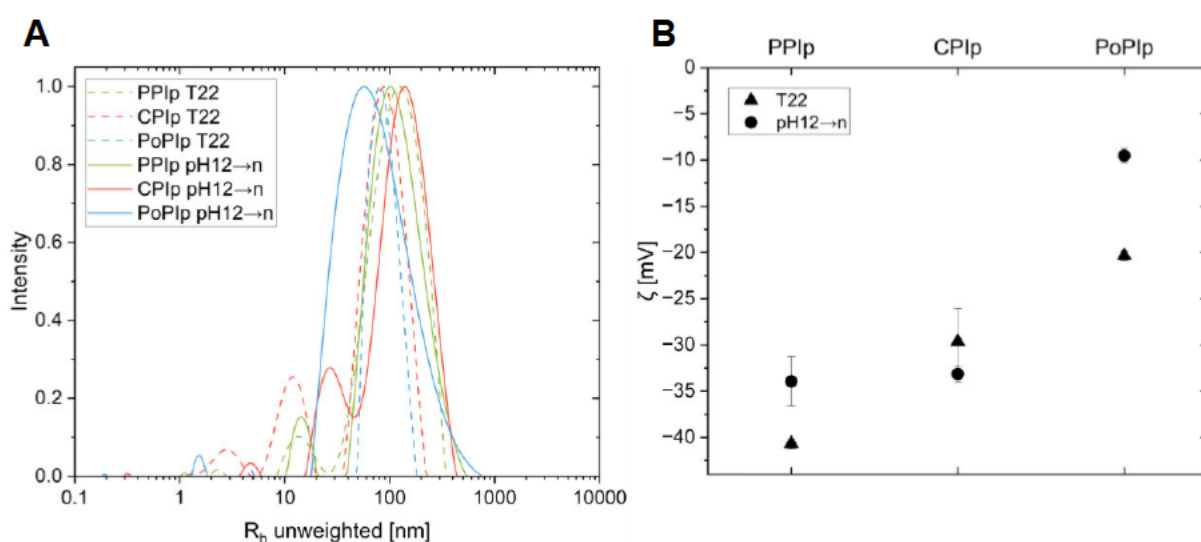


Figure 2.15: Particle size distributions (A) and zeta potentials (ζ , B) of PPIp, CPIp, and PoPIp at T22 (without pH shifting) and after pH shifting (pH12 $\rightarrow$ n).

The two main protein fractions in potato protein isolate, patatin and protease inhibitors, show differing solubilities: patatin has a pI of 4.5-5.2 and is therefore poorly soluble within that pH range^{116,119}. The

pI of patatin coincides with the native pH of the potato protein isolate suspension. Protease inhibitors are a group of proteins with various pIs and pH-dependent solubilities^{19,115,116}. The absolute value of the zeta potential of PoPIp decreases after pH shifting, along with decreasing mean hydrodynamic radii, indicating the formation of smaller, less charged aggregates when the pH is readjusted to the native pH. Although PoPIp's structure changes significantly, the factors limiting solubility seem not to be addressed. Reducing particle size does not increase protein solubility in this case. Therefore, it is suggested that PoPIp may contain fewer ionizable side groups and form smaller hydrophobic particles. The reduced net charge may also suggest that more protease inhibitors are solubilized while less patatin is, shifting the overall pI closer to the native pH and thus decreasing the absolute value of ζ . PPIp and CPIp may contain more ionizable side groups, as they respond to pH shifting similarly to what literature reports (U-shape with a minimum around $pI \approx pH$ 4-6, as reported in several publications^{17,19,81,146}). The greater increase in solubility after basic pH shifting results from stronger electrostatic repulsion caused by abundant glutamic and aspartic acids in pulse proteins, whose side chains deprotonate above pH 4.1 and 3.7, respectively¹⁹. The increase in CPIp's particle size suggests the formation of larger, probably looser, soluble aggregates.

To analyze the impact of basic hydrolysis, NaOH was replaced with the organic base meglumine (Meg). Meg is characterized by bifunctional behavior, acting as both a weak base with $pK_a = 9.53$ and a solubilizing agent¹⁶⁴. Li *et al.* solubilized the pharmaceutical drug glimepiride via hydrogen bond formation with Meg¹⁶⁵. Degot *et al.* and Fusina *et al.* enhanced the solubility of the polyphenols curcumin and quercetin, respectively, via hydrogen bonding with Meg^{164,166}. The change in the base did not affect CPIp solubility but slightly increased that of PPIp and significantly increased that of PoPIp. At pH 8, the equilibrium shifts towards the protonated form of Meg, which no longer acts as a base. Improvements in solubility relative to NaOH can be attributed to hydrogen-bond formation with protein side chains and the addition of a sugar moiety. This supports the hypothesis that PoPIp may be more hydrophobic than PPIp and CPIp, and solubility is increased by making the aggregates more hydrophilic. At pH 10, free hydroxide ions are present in the solution, and no effect on protein solubility is observed. Similar values for PPIp with NaOH and Meg at pH 10 indicate that the strong base and the combined effect of a weak base and the addition of a hydrophilic moiety via hydrogen bonding have comparable solubilization power.

The influence of classical surfactants SDS and CTAB, and the chaotrope urea on the solubility of the protein isolates is discussed in the next section. The results of the solubility screening are presented in Figure 2.16. The particle size distributions and ζ of all protein isolates treated with SDS are shown in Figure 2.17. Protein solubility increases for all isolates with rising SDS concentration (**A**). CPIp solubility rises by factors of 1.2 to 3, reaching 98% at 100 mM SDS. PPIp solubility increases by factors of 2 to 4, reaching 66% at 100 mM SDS. PoPIp solubility increases sevenfold, reaching up to 35% at 100 mM SDS.

The mean hydrodynamic radii decrease at 5 mM SDS below the reference values at T22 and increase significantly at 50 mM SDS to 138, 209, and 104 nm for PPIp, CPIp, and PoPIp, respectively. The absolute values of ζ increase strongly with the addition of SDS. While ζ of PPIp remains unchanged at -46 mV at 5 and 50 mM SDS, ζ of CPIp and PoPIp increase to -40 and -41 mV at 50 mM SDS, respectively. The addition of CTAB has an opposite effect on protein solubility (B). Only CTAB concentrations of 5 and 10 mM can be evaluated for all protein isolates; missing values indicate co-precipitation of the protein isolate and the surfactant. Except for 50 mM CTAB, the solubilities of PPIp and CPIp fall below the reference solubility. PoPIp solubility increases with 5 mM CTAB but then decreases until the protein isolate precipitates.

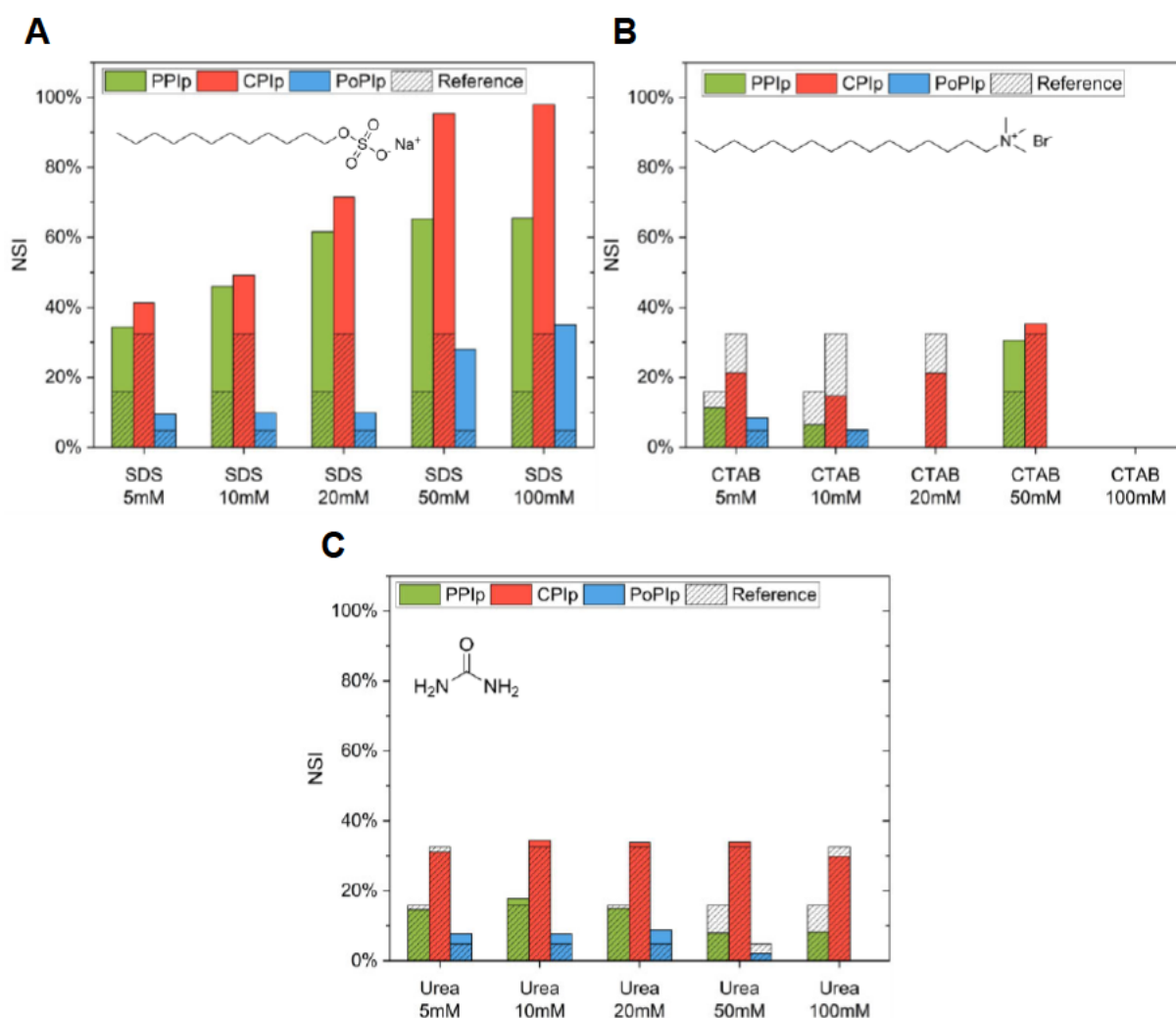


Figure 2.16: Chemical treatment for protein modification. Influence of surfactants SDS (A), CTAB (B), and urea (C) on the aqueous solubility of PPIp, CPIp, and PoPIp. The reference (hatched lines) marks the aqueous solubility at RT without any treatment. The solubility was evaluated at RT, and the respective pH values are summarized in Table SI 2 4.

Adding SDS raises the pH values of the samples (see Table SI 2 4), with pH values ranging from 7.1 to 7.6 for PPIp and CPIp, and from 6.1 to 6.3 for PoPIp. Adding CTAB to the protein suspension lowers the pH of PPIp and CPIp samples to 5.7-6.7. The pH of PoPIp samples decreases to 4.5. Urea addition (C) mainly affects PoPIp solubility, increasing it at 5-20 mM urea, but decreasing it at 50 mM. 100 mM

cannot be assessed due to the co-precipitation of PoPIp and urea. The solubilities of PPIp and CPIp remain nearly unchanged at 5-20 mM (and 50 mM for CPIp) but decline at higher concentrations.

SDS and CTAB are surfactants with a CMC of 8 mM and 0.8 mM, respectively⁴⁸. The CMC decreases in the presence of ions¹⁶⁷. Considering the presence of mineral salts in the isolates, it is assumed that the CMC of SDS dropped below the measured concentration range and that SDS micelles are present throughout the investigated concentration range. SDS is an anionic surfactant, traditionally used as a denaturant in SDS-PAGE to disrupt non-covalent interactions in proteins^{49–51}. The core-shell model of SDS denaturing native proteins discussed in section 2.2.2.5 can also be applied to denatured proteins. The data indicate increased protein solubility through increased surface charge and the formation of potentially detangled aggregates of varying sizes, which enables maximum interaction with SDS monomers and micelles. Particle sizes slightly decrease at a SDS concentration of 5 mM and increase again at 50 mM SDS, indicating a disruption of non-covalent interactions and the formation of heterogeneous, loose structures. According to the literature, 1.4 g of SDS can bind to 1 g of protein⁴⁴. The molar concentration of 5 mM SDS corresponds to a mass concentration of 1.43 mg/mL, facing a protein concentration of 20 mg/mL in the suspension. The molar concentration of 100 mM SDS corresponds to a mass concentration of 28.6 mg/mL. This calculation shows that at 100 mM SDS, there are enough SDS molecules to cover all binding sites in the protein.

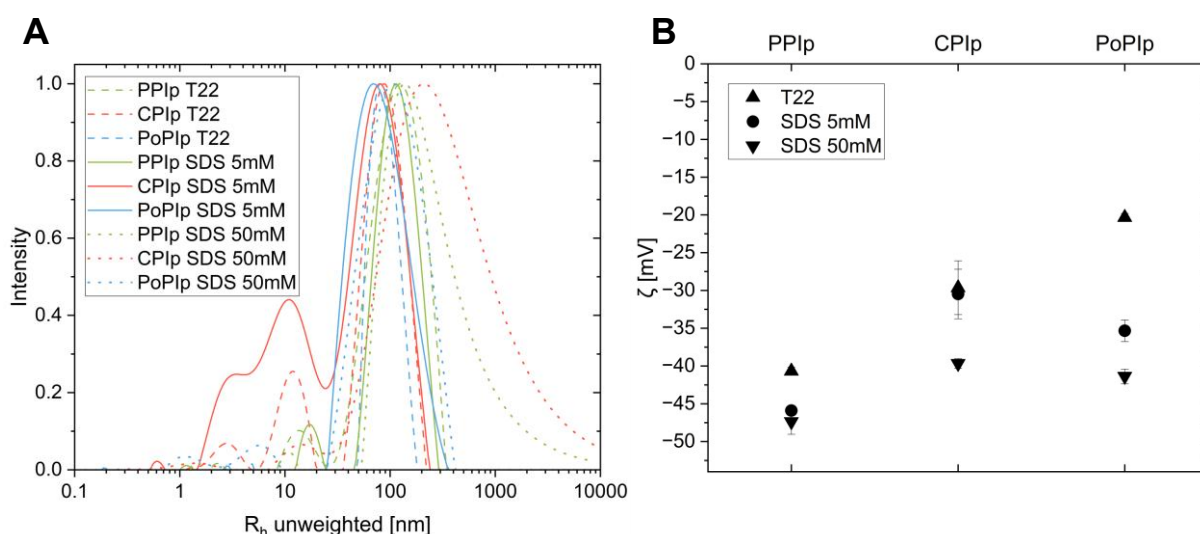


Figure 2.17: Particle size distributions (A) and zeta potentials (ζ , B) of PPIp, CPIp, and PoPIp at T22 (without SDS), at 5 mM SDS, and at 50 mM SDS.

The constant ζ of PPIp with increasing SDS concentration indicates that the number of SDS molecules or micelles bound to the protein remains unchanged, suggesting that not all binding sites are accessible to SDS. The increase in PPIp solubility is more likely due to SDS micelles solubilizing protein fragments in the bulk than to a higher proportion of unfolded protein aggregates. The absolute values of ζ for CPIp and PoPIp increase with rising SDS concentration, suggesting increased interaction between SDS

and proteins. However, PoPIp solubility does not increase as much as that of PPIp and CPIp. This can again be explained by extensive cross-linking and aggregation in the protein isolate, as SDS cannot interrupt covalent bonds^{49–51}.

CTAB is a cationic surfactant with cytotoxic and antiseptic properties, previously used for protein precipitation and still employed for DNA isolation from plants^{48,168}. The significantly reduced solubility of PPIp, CPIp, and PoPIp can be attributed to charge screening when a cationic surfactant is added to a negatively charged protein (pIs between 4.5 and 5.2). Some protease inhibitors of PoPIp have pIs in the basic pH region^{115,116}. Therefore, the increase in solubility at low CTAB concentrations is attributed to increased electrostatic repulsion arising from the protease inhibitors' positive net charge at the investigated pH. The pH of the samples decreases with the addition of CTAB, approaching the pI range of PoPIp and the pH range where PPIp and CPIp exhibit poor solubility. The addition of these two surfactants demonstrated that denatured proteins still respond to the classical trigger of charge screening, and that PoPIp exhibits a higher degree of aggregation than PPIp and CPIp.

Urea is reported to increase protein solubility by breaking hydrogen bonds and disrupting hydrophobic interactions^{44,45}. No effect or salting out of all protein isolates indicates stability or a high number of covalent bonds within the present aggregates. In the literature, high concentrations of urea are commonly used to dissolve proteins^{45,46}. Increasing the concentrations of SCN[−] and urea to 2 M results in the precipitation of the additives, and no NSI can be calculated (data not shown).

Aggregation seems to limit solubility, especially in PoPIp. The most discussed covalent bond is the disulfide bridge formed between two cysteine residues. They can be interrupted by adding a reducing agent, which converts a disulfide bond into two SH groups. Established reducing agents are β -mercaptoethanol and dithiothreitol (DTT). A less harmful and edible reducing agent is Vitamin C¹⁶⁹. The effect of VitC on the solubility of PPIp, CPIp, and PoPIp was investigated and is presented in Figure 2.18. Adding 5 and 10 mM VitC increases PoPIp solubility, reaching a maximum of 14% at 10 mM VitC. The solubilities of PPIp and CPIp exhibit a U-shaped pattern, with a minimum at 20 mM VitC and maxima of 47% and 53%, respectively, at 1.7 M VitC. Adding VitC significantly decreases the pH of PPIp and CPIp samples to 5.8–3.4 at 5–100 mM VitC, and to 2.0 at 1.7 M VitC. For PoPIp, the pH decreases to 4.2–3.0 at 5–100 mM VitC, and to pH 1.8 at 1.7 M VitC (see Table SI 2 5). Unlike its harmful alternatives, VitC is an acid (see the molecular structure in Figure 2.18) with a pK_a of 4.17. The deprotonated form exists at a neutral pH and exhibits the strongest reducing power. In the fully protonated state, its reduction power is diminished¹⁶⁹. The pH decreases with the addition of VitC below the pI of all protein isolates and the pK_a of VitC.

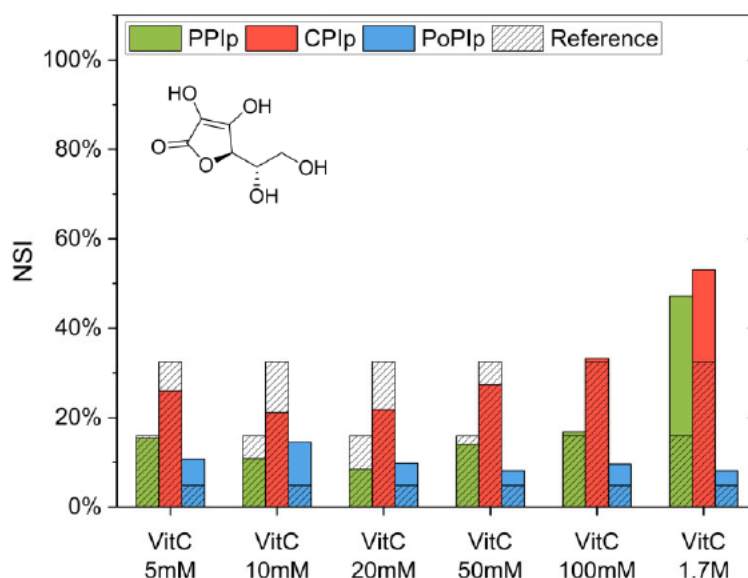


Figure 2.18: Chemical treatment for protein modification. Influence of vitamin C (VitC) on the aqueous solubility of PPIp, CPIp, and PoPIp. The reference (hatched lines) marks the aqueous solubility at RT without any treatment. The solubility was evaluated at RT, and the respective pH values are summarized in Table SI 2 5.

Considering the molecular structures of VitC and the proteins, it is suggested that the increase in solubility of PPIp and CPIp is probably not due to the reduction of covalent bonds; instead, the electrostatic repulsion caused by positively charged protein side groups is likely enhanced by hydrogen bond interactions between the protein side groups and the hydroxy groups of VitC. Another possibility is a Maillard reaction between amine side groups and the hydroxyl group of VitC, representing a non-enzymatic glycation that forms a covalent bond and induces cross-linking. This type of reaction is mainly associated with a decrease in protein solubility, attributed to the cross-linked structure^{170,171}. In the case of denatured protein, interaction with VitC, whether covalent or non-covalent, adds a hydrated moiety to the protein aggregate and could, thus, promote solubility. To exploit the reducing power of VitC, the pH needs to be adjusted to neutral pH. This is carried out in the next section on combined treatments. At low VitC concentrations, the reduced solubility of PPIp and CPIp is probably attributed to the zero net charge of the proteins, caused by the pI in that pH range. In the case of PoPIp, solubility peaks at 10 mM VitC. The sample's pH is close to 4. Consequently, the equilibrium shifts towards the protonated form of PoPIp ($\text{pH} < \text{pI}(\text{PoPIp})$) and towards the neutral form of VitC ($\text{pH} \approx \text{pK}_a(\text{VitC})$). An increase in electrostatic repulsion within the protein, combined with an enhanced reducing capacity of VitC, could lead to increased covalent bond breaking within the protein due to improved access. The increased solubility mainly favors the latter. Due to the limited number of amine side groups in potato protein, increasing the concentration of VitC does not favor the Maillard reaction^{171,172}.

2.4.2.3. Combined treatments

The solubilization capacities of both common and less used modification methods on commercially available, denatured protein isolates were examined in the previous chapter. This chapter focuses on systematically combining different solubilization mechanisms to identify and evaluate potential interactions and synergistic effects.

The combinations of pH shifting (pH12→n) with heating and pH shifting (pH12→n) with sonication involve both chemical and physical treatments. The solubilities are shown in Figure 2.19. The combination of pH shifting and heating significantly increases the protein solubility of all isolates. The most notable increase occurs at 80 °C, with solubilities reaching 95-96% for PPIp and CPIp, and 22% for PoPIp. Compared to pH shifting alone (see Figure 2.14) and heating alone (see Figure 2.11), pH/T significantly increases the water solubility of all protein isolates.

Combining pH shifting with sonication also enhances the protein solubility of all isolates. For PPIp, the most notable increase occurs when the pH is raised to 12, followed by sonication, and then the pH is readjusted to its native level, resulting in about 80% solubility. CPIp exhibits a U-shaped solubility pattern, with maximum solubility around 50% at highly acidic or alkaline pH levels. PoPIp solubility only increases to 9-11%, regardless of pH. Compared with treatments applied separately, pH/US protein solubility increases for all isolates (except for CPIp at pH12→n).

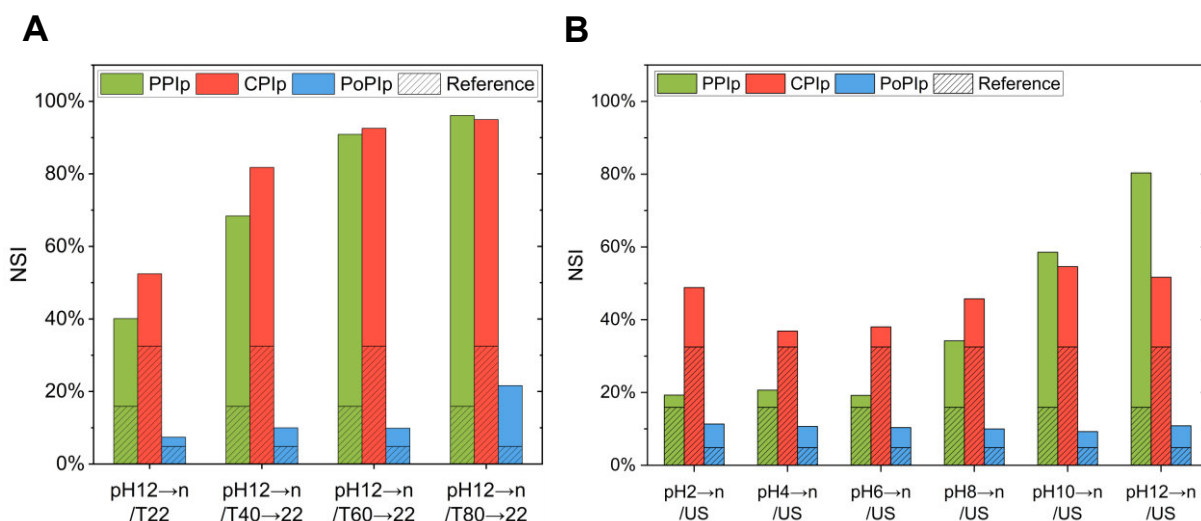


Figure 2.19: Combined treatment for protein modification. Influence of pH shifting and heating (A) and pH shifting and sonication (B) on the aqueous solubility of PPIp, CPIp, and PoPIp. The reference (hatched lines) marks the aqueous solubility at RT without any treatment. The solubility was evaluated at RT and native pH.

In both cases, the physical triggers are applied after adjusting the pH to 12, ensuring a less rigid protein aggregate structure. While sonication affects non-covalent interactions, changes the secondary and tertiary structures, and is mainly used to reduce particle size, heating in the presence of hydroxide ions can promote more extensive unfolding and/or basic hydrolysis^{17,19,83,140}. Jiang *et al.* and Lee *et al.*

studied the influence of pH shifting and sonication on a NUTRALYS® pea protein isolate from Roquette and two commercial soy protein isolates, respectively. Both observed a significant increase in protein water solubility with the combined treatment, attributed to particle size reduction resulting from the disruption of covalent and non-covalent bonds. Additionally, surface hydrophobicity was increased^{173,174}. Both modification combinations are used to functionalize pulse proteins for the solubilization of nutraceuticals, such as polyphenols or vitamins^{68,174,175}.

VitC can act as a glycation or reducing agent, as discussed in the previous section. To investigate both modification pathways more deeply, it was combined with sonication, pH shifting, and heating. The solubilities are presented in Figure 2.20. The effect of VitC at $c(\text{VitC}) = 1.7 \text{ M}$ combined with sonication is assessed in two different sequences. Both enhance the protein solubility of all isolates compared to treatments with only sonication (see Figure 2.11) and only VitC (see Figure 2.18). When PPIp, CPIp, and PoPIp are first suspended in a VitC solution with a pH of 1.8 to 2.0 (see Table SI 2 6), and then subjected to sonication, higher solubilities are achieved compared to the reverse order, reaching 86% for PPIp, 70% for CPIp, and 14% for PoPIp.

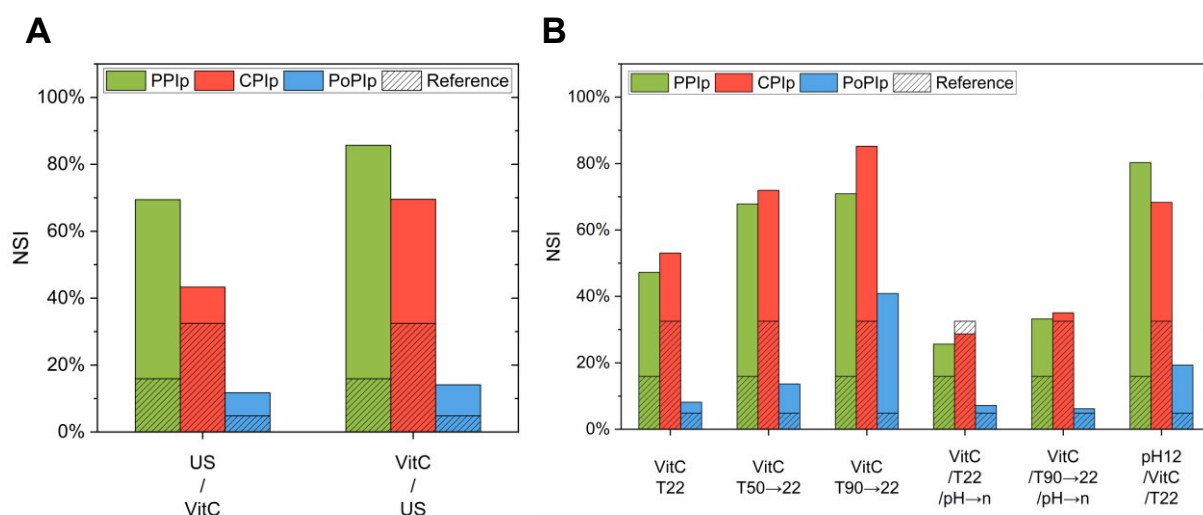


Figure 2.20: Combined treatment for protein modification. Influence of sonication and VitC ($c = 1.7 \text{ M}$) in different orders (**A**), VitC and heating (**B left**) and VitC combined with pH shifting and heating (**B right**) on the solubility of PPIp, CPIp, and PoPIp. The reference (hatched lines) marks the aqueous solubility at RT without any treatment. The solubility was evaluated at RT, and the respective pH values are summarized in Table SI 2 6.

The effect of VitC is also combined with heating and pH shifting in various sequences. Heating to 90°C for 10 minutes, which resembles pasteurization conditions¹⁷⁶, further increases solubility, especially for PoPIp, compared to heating to 50°C for two hours. Solubilities of 71% for PPIp, 85% for CPIp, and 41% for PoPIp can be achieved. When readjusting the pH to its native level after stirring at RT or heating, the solubility drops significantly. Adjusting to pH 12, adding VitC, which lowers the pH to 1.8-2.0, and stirring at RT for two hours achieves solubilities comparable to those obtained by combining VitC and sonication.

In case **A**, VitC exists in its protonated form and has a decreased reduction potential. When combined with sonication, the increased protein solubility can be attributed to electrostatic repulsion between protein side groups and extensive glycation, which may be facilitated by the reduction in aggregate size. In case **B**, it should be considered that VitC is a labile compound that degrades rapidly in alkaline aqueous systems and above room temperature¹⁶⁹. Comparing the effect of pH 2 adjusted with VitC and pH 2 adjusted with HCl (see Figure 2.14) and evaluating the solubility after readjustment to the native pH, no effect of VitC can be observed. Therefore, the increase in solubility at 1.7 M VitC and heating can be attributed to the acidic pH and electrostatic repulsion. The same conclusion can be drawn for the addition of VitC after adjusting to pH 12. The only exception is PoPlp, whose protein solubility can be significantly increased by heating compared to VitC at RT (see Figure 2.18).

Experiments combining VitC with physical treatments and pH control indicate that, for the tested protein isolates, electrostatic repulsion at acidic pH outweighs the reducing and glycation effects of VitC.

VitC was combined with SDS to assess potential synergies between electrostatic and glycation effects and solubilization of hydrophobic protein regions. VitC and SDS were mixed in two different sequences with $c(\text{VitC}) = 1.7 \text{ M}$ and $c(\text{SDS})$ ranging from 5 to 100 mM. The solubilities are shown in Figure 2.21.

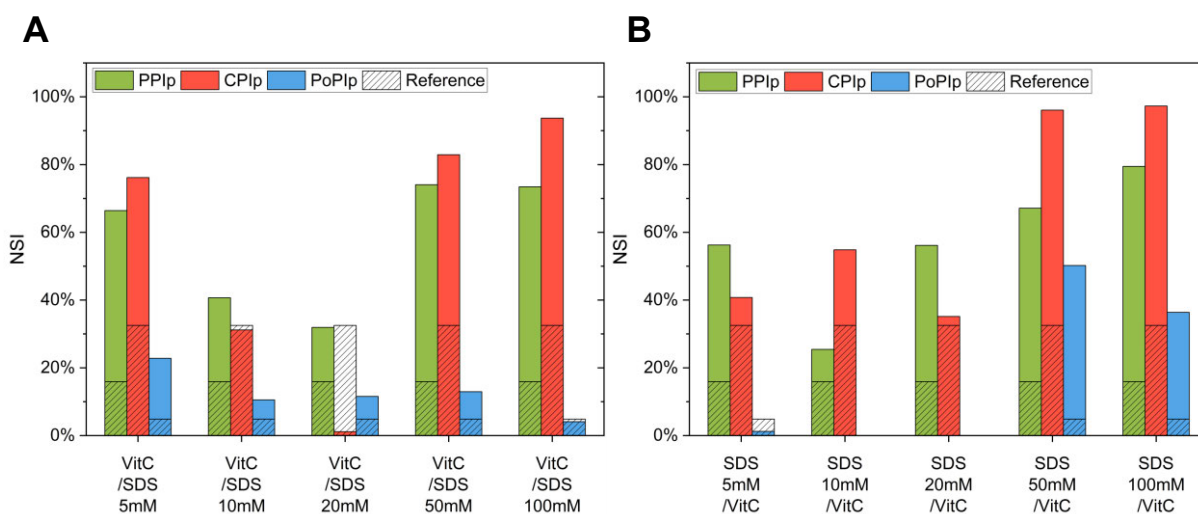


Figure 2.21: Combined treatment for protein modification. Influence of SDS and VitC ($c = 1.7 \text{ M}$) in different orders on the solubility of PPIp, CPIp, and PoPlp. The reference (hatched lines) marks the aqueous solubility at RT without any treatment. The solubility was evaluated at RT, and the respective pH values are summarized in Table SI 2 6.

Comparing solubilities from combined treatments with those from separate treatments shows no significant synergistic effect. In both cases, either the SDS influence dominates, producing values similar to those of pure SDS treatment, or the VitC influence dominates, resulting in solubilities similar to those of pure VitC treatment. A combined effect – such as increased hydrophilicity via glycation by VitC and aggregate loosening and solubilization of hydrophobic patches through interaction with SDS – is only observed in specific cases, such as PoPlp at 50 mM SDS in **A**. However, a negative synergistic

effect is mainly observed, leading to the co-precipitation of the compounds present. The pH remains around 2 in both experiments. Due to SDS's molecular structure, protonation at low sample pH can be neglected. Therefore, electrostatic screening of positively charged protein side chains and negatively charged SDS molecules or micelles may explain the co-precipitation.

Especially for PoPIp, using SDS offers the most promising approach to increase protein solubility. Similar to VitC, SDS should also be examined more thoroughly to enhance its effect by combining it with other methods. The solubilities of SDS when combined with heating and sonication are shown in Figure 2.22. Heating the SDS suspension above 40 °C increases the solubility of PPIp and PoPIp compared to RT, with PPIp reaching nearly complete solubility and PoPIp reaching 70% (A). CPIp remains highly soluble across all temperatures. Sonication does not significantly affect the solubility of CPIp and PoPIp but slightly improves PPIp solubility across all SDS concentrations compared to SDS treatment alone (see Figure 2.16), reaching 84% at 50-100 mM SDS (B).

The increase in PPIp solubility appears to be an additive effect of SDS and sonication. For PoPIp, a synergy between SDS and heating can be observed. By disrupting the protein aggregates with heating, the interactions with SDS may be enhanced.

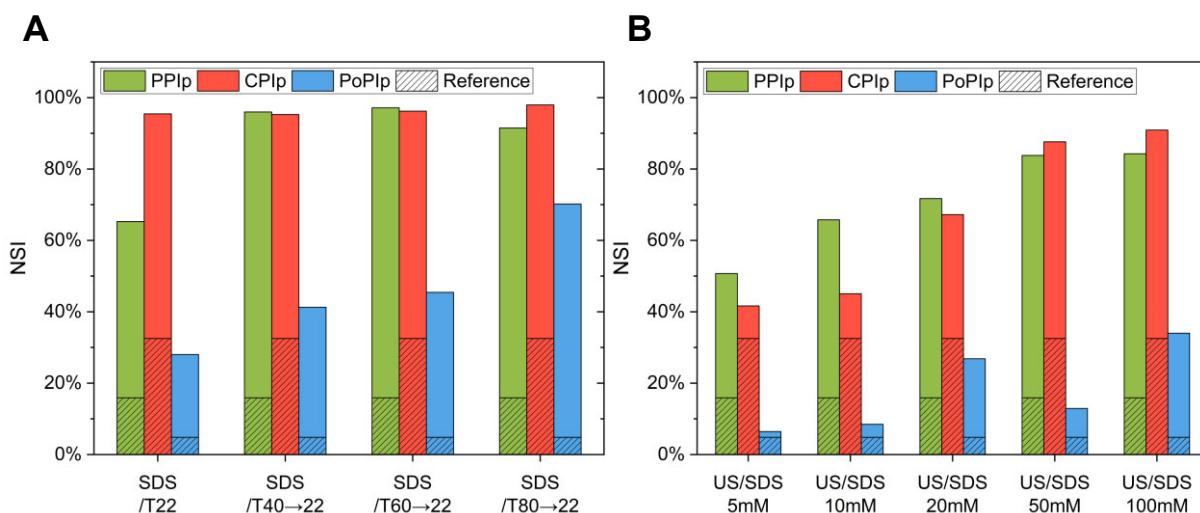


Figure 2.22: Combined treatment for protein modification. Influence of 50 mM SDS and heating (A) and 5-100 mM SDS and sonication (B) on the solubility of PPIp, CPIp, and PoPIp. The reference (hatched lines) marks the aqueous solubility at RT without any treatment. The solubility was evaluated at RT. The pH values were assumed to be similar to suspensions of protein isolates and SDS (see Table SI 2 4).

The combination of SDS and heating nearly fully solubilized PPIp and CPIp. For PoPIp, it appears essential to improve the availability of interaction sites with SDS. Therefore, the effects of pH shifting, as well as heating combined with pH shifting, were tested to ensure maximum disruption of aggregates. Generally, shifting to a highly alkaline pH showed a greater solubility-enhancing effect than shifting to an acidic pH (see Figure 2.14). The protein solubilities of all isolates subjected to pH shifting, combined with SDS addition and heating, are presented in Figure 2.23.

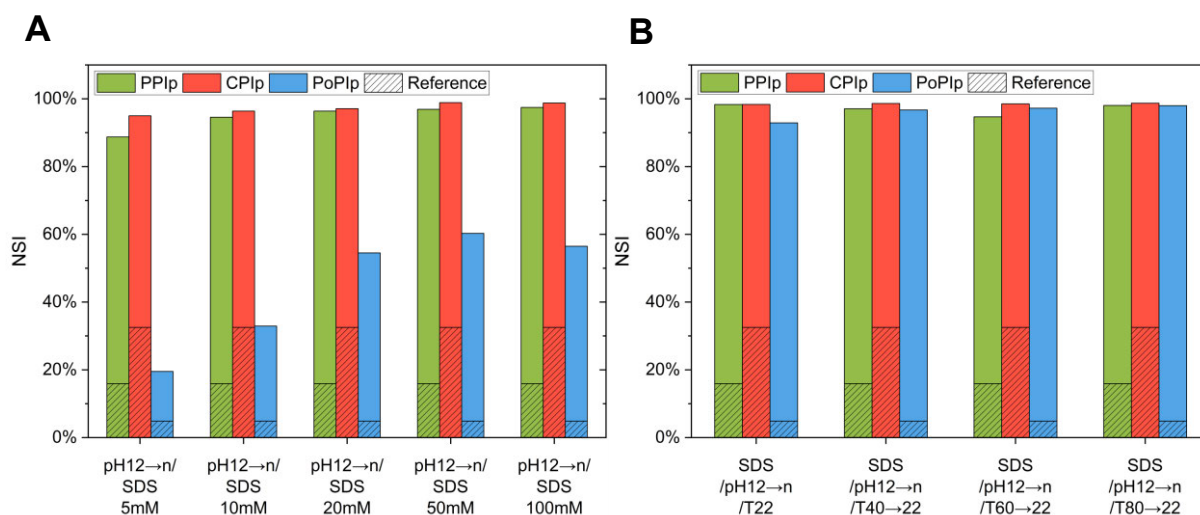


Figure 2.23: Combined treatment for protein modification. Influence of pH shifting and 5-100 mM SDS (**A**) and 50 mM SDS, pH shifting and heating (**B**) on the solubility of PPIp, CPIp, and PoPip. The reference (hatched lines) marks the aqueous solubility at RT without any treatment. The solubility was evaluated at RT and native pH.

Unfolding the protein first by adjusting to pH 12, then adding SDS, and readjusting to native pH (**A**) increases the protein solubility of all isolates. In the case of PPIp and CPIp, it appears to be an additive effect of pH shifting and SDS (see Figure 2.14 and Figure 2.16, respectively), reaching nearly complete solubilization. For PoPip, pH shifting enhances the interaction between SDS and the protein aggregates, boosting the solubility to 60% and thus exceeding the results obtained with separate treatments. Reversing the order of SDS and pH shifting (**B**) results in the complete solubilization of all isolates. Heating is only necessary for PoPip if the goal is to increase protein solubility from 93% to 98%.

The hydrodynamic radii slightly increase for SDS/pH12→n/T22 and then decrease again for SDS/pH12→n/T80→22 (see Figure 2.24, **A**) compared to the reference at T22. Hydrodynamic radii of 114, 65, and 99 nm are observed for PPIp, CPIp, and PoPip, respectively, for SDS/pH12→n/T80→22. This means a slight decrease in particle sizes for PPIp and CPIp and an increase for PoPip, compared to the reference at T22. The absolute ζ values remain nearly unchanged when combining 50 mM SDS with pH shifting (**B**) (except for an increase of ζ for PPIp) compared to 50 mM SDS without pH shifting (see Figure 2.17). When combined with T80→22, these values increase to -65, -50, and -32 mV for PPIp, CPIp, and PoPip, respectively. SDS-PAGEs of the solutions (**C**) show blurred bands for PPIp and CPIp covering the entire gel. This may indicate a reorganization of aggregated protein fractions. The same pattern is observed with PoPip, indicating the solubilization of protein fractions at that mass range compared to no solubilization in the reference state. The increased intensity at very low kDa (<15 kDa) for all protein isolates indicates significant fragmentation of protein aggregates caused by the treatment. The obtained solutions are clear to opalescent (**D**).

Combining SDS-protein interaction, SDS-mediated solubilization of hydrophobic regions, and destabilization of protein aggregates via pH shifting yielded a consistent method for fully solubilizing the protein content across all isolates. Although the effects of each compound are well documented in the literature, the combined use of both in that order has not been reported according to the current state of research.

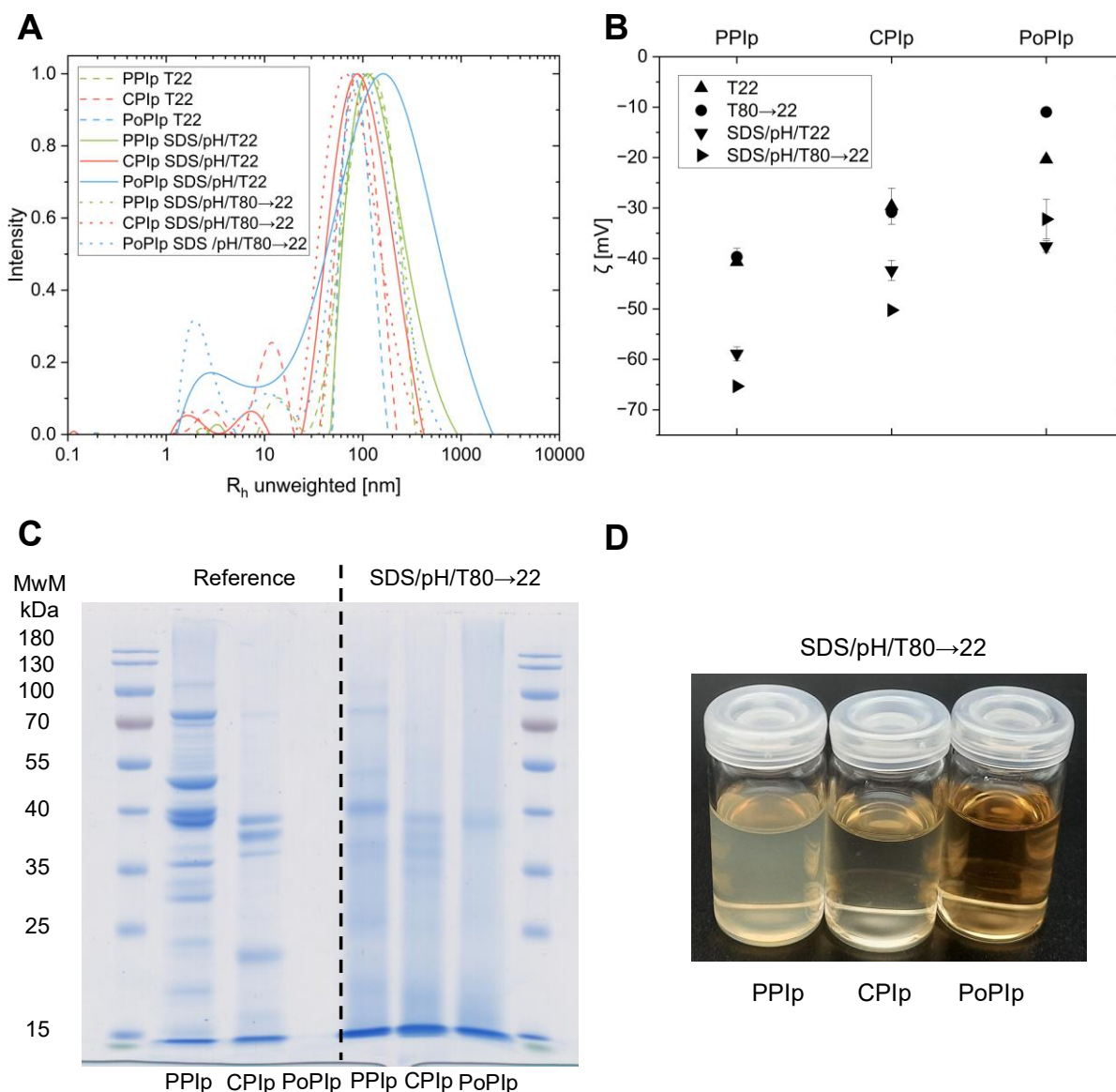
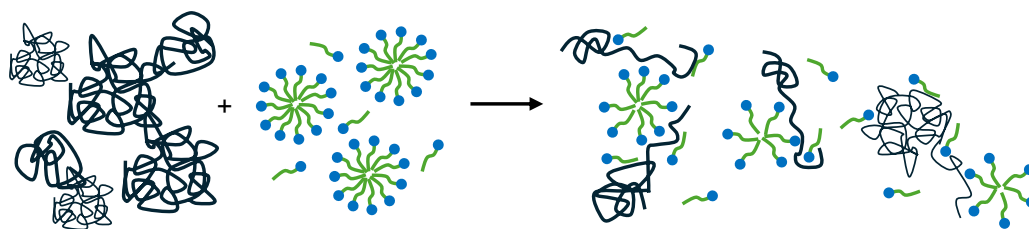


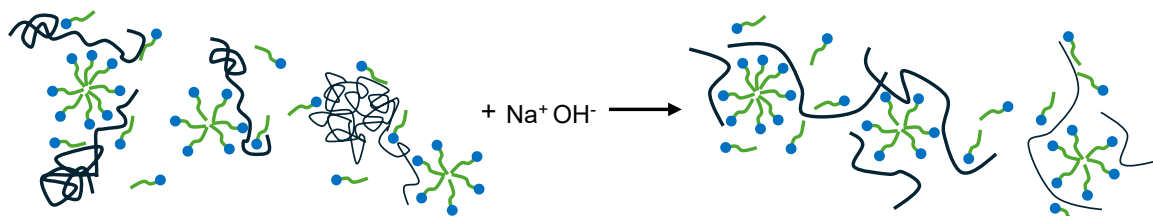
Figure 2.24: Particle size distributions (**A**), zeta potentials (ζ , **B**), SDS-PAGE (**C**), and photo (**D**) of PPIp, CPIp, and PoPIp at SDS/pH/T80→22.

Compared to the reference at T22, the particle sizes decreased for PPIp and CPIp and increased for PoPIp. The absolute ζ values increased significantly, suggesting a strong interaction between SDS molecules and micelles with the proteins. A schematic solubilization mechanism is illustrated in Figure 2.25. The first step builds on the explanation of SDS's influence on denatured proteins, as previously described with the SDS concentration series (Figure 2.16 A).

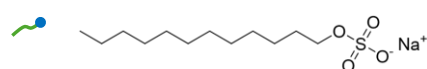
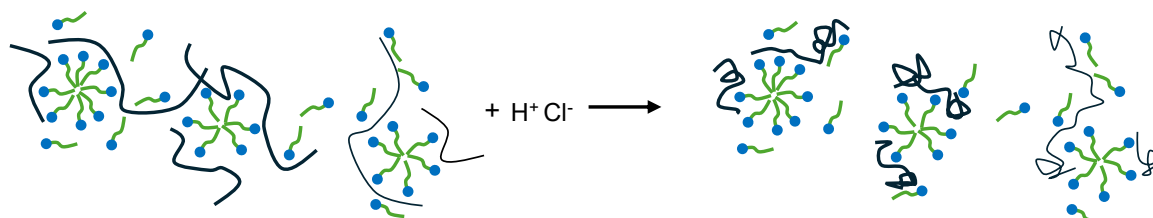
1. Suspension of protein isolate in SDS solution at $c(\text{SDS}) = 50\text{mM}$:



2. pH shifting to pH 12 using NaOH:



3. pH shifting to native pH using HCl:



 Protein isolate aggregates

Figure 2.25: Schematic representation of the solubilization of protein aggregates by combining SDS and pH shifting.

Protein aggregates are loosened to maximize favorable interactions with SDS micelles and monomers through electrostatic and hydrophobic interactions. Here, the SDS-to-protein mass ratio is 0.72, calculated from the suspension's mass concentrations. When the amount of protein exceeds SDS, it can be assumed that one SDS micelle is coated with different proteins, forming a core-shell structure⁴⁹. The protein aggregates are only partially loosened and unfolded, and not entirely in contact with SDS. Upon adding NaOH, additional electrostatic repulsion is induced by deprotonation of ionizable side groups, leading to further unfolding or partial hydrolysis of the protein. This promotes increased contact between SDS and proteins. By readjusting the pH to the native level, electrostatic repulsion is minimized, and proteins re-aggregate into smaller particles in contact with SDS. This process enables solubility at the native pH. PoPIp behaved differently during the experiments compared to PPIp and CPIp. Having identified a consistent approach, a deeper understanding of the solubility-limiting factors of various commercial plant protein products is gained, summarizing them into two critical aspects: the protein's hydrophobicity and the disruption of aggregates caused by processing and extraction.

2.4.2.4. Summary

A series of influencing factors and solubilization mechanisms was tested on a selection of commercially available protein isolates. Table 2.4 provides an overview of protein solubility for different treatments. When the parameters within one treatment category were varied, the one achieving the highest solubility is indicated in brackets below the % (w/w).

Table 2.4: Overview of protein solubilities of PPIp, CPIp, and PoPIp after different treatments. The solubilities are given in % (w/w). The treatment conditions achieving the highest solubility are shown in brackets below the solubility.

Treatment	Protein solubility [%]		
	PPIp (Treatment condition)	CPIp (Treatment condition)	PoPIp (Treatment condition)
Reference H ₂ O	16	33	5
T	64 (T 80→22)	39 (T 80→22)	6 (T 60→22)
US	31	35	5
Degas	16	28	8
Hofmeister series	19 (NaCl 5 mM)	35 (NaCl 5 mM)	7 (NaSCN 5 mM)
pH-shift	40 (pH 12→n, NaOH)	52 (pH 12→n, NaOH)	9 (pH 8→n, Meg)
Surfactants	66 (SDS 100 mM)	98 (SDS 100 mM)	35 (SDS 100 mM)
Urea	18 (10 mM)	34 (10 mM)	9 (20 mM)
VitC	47 (1.7 M)	53 (1.7 M)	10 (100 mM)
pH/T	96 (T 80→22)	95 (T 80→22)	22 (T 80→22)
pH/US	80 (pH 12→n)	55 (pH 10→n)	11 (pH 2→n)
VitC/US	86	70	14
VitC/T/pH	80 (pH 12/T 22)	85 (T 90→22)	41 (T 90→22)
SDS/VitC	79 (SDS 100 mM)	97 (SDS 100 mM)	50 (SDS 50 mM)
SDS/T	97 (T 60→22)	98 (T 80→22)	70 (T 80→22)
US/SDS	84 (SDS 100 mM)	91 (SDS 100 mM)	34 (SDS 100 mM)
SDS/pH/(+T)	98 (T22)	99 (T22-80→22)	98 (T>40→22)

The protein solubilities of PPIp and CPIp behaved similarly, while that of PoPIp exhibited distinct behavior. PPIp and CPIp responded positively to various solubilization factors, whereas PoPIp reacted inertly to many of these factors. Characterization of the protein isolates (section 2.4.1) and experiments with Hofmeister salts (Figure 2.13) indicated a highly denatured state for all protein isolates, regardless of suppliers. PPIp and CPIp are pulse proteins with similar compositions; thus, similar solubilization behavior was expected. Protein solubility is determined by covalent and non-covalent interactions, influenced by a range of external parameters (see Figure 2.4).

Physical treatments do not affect covalent bonds. The protein solubility of all isolates remained low after these treatments, indicating that covalent bonds and aggregation limit solubility. pH shifting and VitC influence both covalent and non-covalent interactions by causing electrostatic repulsion and modifying the primary structure through hydrolysis and glycation. The reduction potential of VitC was challenging to assess and was considered low in these experiments. Combining both factors with heating significantly enhanced their solubilization power (Figure 2.19 **A**, Figure 2.20 **B**). Adding 100 mM SDS considerably increased the solubility of CPIp and PoPIp, while urea had the opposite effect (Figure 2.16 **A, C**). Both additives impact non-covalent interactions; however, SDS introduces an ionic component along with hydrogen bonding and hydrophobic interactions (discussed in detail in section 2.2.2.5). The effect of SDS could be further enhanced by heating, leading to nearly complete solubilization of PPIp and CPIp (Figure 2.22 **A**). To fully solubilize PoPIp, the effect of SDS was combined with pH shifting (and heating), probably reaching full access to the denatured and stable aggregates (Figure 2.23 **B**). Particle size and zeta potential measurements of the single steps in the combined approach, which achieved complete protein solubilization, showed that protein solubility depends on a combination of various factors rather than single parameters. For commercial protein isolates, it seems to be the interplay between tackling the hydrophobicity of the protein and disrupting aggregates caused by processing and extraction.

2.5. Conclusion and Outlook

Plant proteins provide an appealing and essential alternative to animal proteins, not only for nutrition and global welfare, but also concerning their techno-functional properties. However, these properties are often difficult to access or have been diminished due to denaturing extraction methods. Currently, research focuses on developing milder extraction techniques and scaling them up to industrial levels. Another extensively explored area is the functionalization of existing products. In this chapter, various physical and chemical modification methods, both alone and in combination, were applied to commercially available plant protein isolates to influence their aqueous solubility. The solubilities were evaluated as relative values by calculating the NSI. The aqueous protein solubilities of PPIp, CPIp, and PoPIp were 16%, 32%, and 5% (w/w), respectively. The relatively low protein solubility, along with DSC, TGA, and SDS-PAGE data, indicated a significantly denatured state of the proteins. Both PPIp and CPIp are pulse protein isolates and behaved similarly in the solubility experiments due to their similar protein composition. PoPIp exhibited different behavior and was less responsive to solubility-enhancing treatments. By combining external triggers that target non-covalent and covalent bonds, all protein isolates could be solubilized. This was achieved by preparing a protein isolate suspension in a 50 mM SDS solution, then adjusting to pH 12 and optionally heating. After cooling to room temperature, the pH was readjusted to its native level. The combination of SDS with pH shifting ensured the destabilization of insoluble aggregates through protein unfolding and increased interaction with SDS. DLS, zeta potential measurements, and SDS-PAGE revealed strongly negatively charged aggregates with mean hydrodynamic radii of 114 nm for PPIp, 65 nm for CPIp, and 99 nm for PoPIp. The particle size decreased due to protein re-aggregation. The experiments presented were conducted as a screening study, with parameter optimization only within a limited scope. Regarding the approach $\text{SDS/pH12} \rightarrow \text{n/T}$, the SDS concentration or the alkaline pH could be optimized. Additionally, requirements differ depending on the application. Despite being performed in an industrial context with corresponding requirements, these experiments are not targeted at a specific application but rather provide insights into solubilization mechanisms. By contributing to a deeper understanding of the behavior of commercial plant protein isolates, their roles in nutrition and technology can be advanced. At the same time, it is emphasized that shifting the focus in extraction from protein mass to protein functionality is essential. The investigation and targeted modification of protein solubility, however, extend beyond the immediate field of application presented here and become evident in more complex contexts, such as those related to neurodegenerative diseases.

2.6. References

- (1) Wieland T., B. M. *The World of Peptides: A Brief History of Peptide Chemistry*; Springer: Berlin Heidelberg, **1991**. ISBN 3-540-52830-X.
- (2) Alberts, B.; Heald, R.; Johnson, A.; Morgan, D.; Raff, M.; Roberts, K.; Walter, P., Eds. *Molecular Biology of the Cell*, seventh edition; W. W. Norton & Company: New York, **2022**. ISBN 9780393884821.
- (3) Nobel Prize Outreach 2025. The Nobel Prize in Chemistry 1962. <https://www.nobelprize.org/prizes/chemistry/1962/summary> (accessed Dec 3, 2025).
- (4) Dyson, H. J.; Wright, P. E.; Scheraga, H. A. The role of hydrophobic interactions in initiation and propagation of protein folding. *PNAS* **2006**, *103*, 13057–13061. <https://doi.org/10.1073/pnas.0605504103>.
- (5) Gromiha, M. M.; Selvaraj, S. Inter-residue interactions in protein folding and stability. *Prog Biophys Mol Biol* **2004**, *86*, 235–277. <https://doi.org/10.1016/j.pbiomolbio.2003.09.003>.
- (6) Gallivan, J. P.; Dougherty, D. A. A Computational Study of Cation– π Interactions vs Salt Bridges in Aqueous Media: Implications for Protein Engineering. *J. Am. Chem. Soc.* **2000**, *122*, 870–874. <https://doi.org/10.1021/ja991755c>.
- (7) Kumar, S.; Nussinov, R. Salt Bridge Stability in Monomeric Proteins. *J. Mol. Biol.* **1999**, *293*, 1241–1255. <https://doi.org/10.1006/jmbi.1999.3218>.
- (8) Ghosh, D.; Biswas, A.; Radhakrishna, M. Advanced computational approaches to understand protein aggregation. *Biophys Rev* **2024**, *5*, 021302-1-021302-40. <https://doi.org/10.1063/5.0180691>.
- (9) Jahn, T. R.; Radford, S. E. The Yin and Yang of protein folding. *FEBS J.* **2005**, *272*, 5962–5970. <https://doi.org/10.1111/j.1742-4658.2005.05021.x>.
- (10) Teilum, K.; Olsen, J. G.; Kragelund, B. B. Protein stability, flexibility and function. *Biochim. Biophys. Acta - Proteins Proteom.* **2011**, *1814*, 969–976. <https://doi.org/10.1016/j.bbapap.2010.11.005>.
- (11) Silva, J. L.; Vieira, T. C. R. G.; Gomes, M. P. B.; Bom, A. P. A.; Lima, L. M. T. R.; Freitas, M. S.; Ishimaru, D.; Cordeiro, Y.; Foguel, D. Ligand binding and hydration in protein misfolding: insights from studies of prion and p53 tumor suppressor proteins. *Acc. Chem. Res.* **2010**, *43*, 271–279. <https://doi.org/10.1021/ar900179t>.
- (12) Mehringer, J. Stability of Proteins in the Presence of Anionic Additives. PhD Thesis, University of Regensburg, Regensburg, **2021**. <http://doi.org/10.5283/epub.51476>.
- (13) Gomes, C. M. Protein misfolding in disease and small molecule therapies. *Curr. Top. Med. Chem.* **2012**, *12*, 2460–2469. <https://doi.org/10.2174/1568026611212220002>.
- (14) Wang, W.; Nema, S.; Teagarden, D. Protein aggregation—Pathways and influencing factors. *Int. J. Pharm.* **2010**, *390*, 89–99. <https://doi.org/10.1016/j.ijpharm.2010.02.025>.

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- (15) Lashuel, H. A. Rethinking protein aggregation and drug discovery in neurodegenerative diseases: Why we need to embrace complexity? *Curr Opin Chem Biol* **2021**, *64*, 67–75. <https://doi.org/10.1016/j.cbpa.2021.05.006>.
- (16) Sheikh, S.; Safia; Haque, E.; Mir, S. S. Neurodegenerative Diseases: Multifactorial Conformational Diseases and Their Therapeutic Interventions. *J. Neurodegener. Dis.* **2013**, *2013*, 563481-563489. <https://doi.org/10.1155/2013/563481>.
- (17) Gao, K.; Rao, J.; Chen, B. Plant protein solubility: A challenge or insurmountable obstacle. *Adv. Colloid Interface Sci.* **2024**, *324*, 103074–103088. <https://doi.org/10.1016/j.cis.2023.103074>.
- (18) Wang, Z.; Lan, T.; Jiang, J.; Song, T.; Liu, J.; Zhang, H.; Lin, K. On the modification of plant proteins: Traditional methods and the Hofmeister effect. *Food Chem.* **2024**, *451*, 139530–139539. <https://doi.org/10.1016/j.foodchem.2024.139530>.
- (19) Grossmann, L.; McClements, D. J. Current insights into protein solubility: A review of its importance for alternative proteins. *Food Hydrocoll.* **2023**, *137*, 108416–108437. <https://doi.org/10.1016/j.foodhyd.2022.108416>.
- (20) Pelegri, D.; Gasparetto, C. A. Whey proteins solubility as function of temperature and pH. *LWT* **2005**, *38*, 77–80. <https://doi.org/10.1016/j.lwt.2004.03.013>.
- (21) Osborne, T. B. *The vegetable proteins*; Longmans: London, **1909**.
- (22) Lam, A. C. Y.; Can Karaca, A.; Tyler, R. T.; Nickerson, M. T. Pea protein isolates: Structure, extraction, and functionality. *Food Rev. Int.* **2018**, *34*, 126–147. <https://doi.org/10.1080/87559129.2016.1242135>.
- (23) Michaelis, L.; Macinnes, D. A.; Granick, S. *Leonor Michaelis: A Biographical Memoir*; National Academy of Sciences: Washington D.C., **1958**.
- (24) Righetti, P. G. Determination of the isoelectric point of proteins by capillary isoelectric focusing. *J. Chromatogr. A* **2004**, *1037*, 491–499. <https://doi.org/10.1016/j.chroma.2003.11.025>.
- (25) Salgin, S.; Salgin, U.; Bahadır, S. Zeta Potentials and Isoelectric Points of Biomolecules: The Effects of Ion Types and Ionic Strengths. *Int. J. Electrochem. Sci.* **2012**, *7*, 12404–12414. [https://doi.org/10.1016/S1452-3981\(23\)16554-0](https://doi.org/10.1016/S1452-3981(23)16554-0).
- (26) Karabulut, G.; Goksen, G.; Mousavi Khaneghah, A. Plant-based protein modification strategies towards challenges. *J. Agric. Food Res.* **2024**, *15*, 101017–101031. <https://doi.org/10.1016/j.jafr.2024.101017>.
- (27) Hofmeister, F. Zur Lehre von der Wirkung der Salze. *Arch. Exp. Pathol. Pharmacol.* **1888**, *25*, 1–30. <https://doi.org/10.1007/BF01838161>.
- (28) Kunz, W.; Henle, J.; Ninham, B. W. 'Zur Lehre von der Wirkung der Salze' (About the science of the effect of salts): Franz Hofmeister's Historical Papers. *Curr. Opin. Colloid Interface Sci.* **2004**, *9*, 19–37. <https://doi.org/10.1016/j.cocis.2004.05.005>.
- (29) Collins, K. D. Ions from the Hofmeister series and osmolytes: effects on proteins in solution and in the crystallization process. *Methods* **2004**, *34*, 300–311. <https://doi.org/10.1016/j.ymeth.2004.03.021>.

- (30) Washabaugh, M. W.; Collins, K. D. The systematic characterization by aqueous column chromatography of solutes which affect protein stability. *J. Biol. Chem.* **1986**, *261*, 12477–12485. [https://doi.org/10.1016/S0021-9258\(18\)67112-1](https://doi.org/10.1016/S0021-9258(18)67112-1).
- (31) Gregory, K. P.; Elliott, G. R.; Robertson, H.; Kumar, A.; Wanless, E. J.; Webber, G. B.; Craig, V. S. J.; Andersson, G. G.; Page, A. J. Understanding specific ion effects and the Hofmeister series. *Phys. Chem. Chem. Phys.* **2022**, *24*, 12682–12718. <https://doi.org/10.1039/D2CP00847E>.
- (32) Lo Nostro, P.; Ninham, B. W. Hofmeister phenomena: an update on ion specificity in biology. *Chem. Rev.* **2012**, *112*, 2286–2322. <https://doi.org/10.1021/cr200271j>.
- (33) Okur, H. I.; Hladílková, J.; Rembert, K. B.; Cho, Y.; Heyda, J.; Dzubiella, J.; Cremer, P. S.; Jungwirth, P. Beyond the Hofmeister Series: Ion-Specific Effects on Proteins and Their Biological Functions. *J. Phys. Chem. B.* **2017**, *121*, 1997–2014. <https://doi.org/10.1021/acs.jpcb.6b10797>.
- (34) Kunz, W.; Lo Nostro, P.; Ninham, B. W. The present state of affairs with Hofmeister effects. *Curr. Opin. Colloid Interface Sci.* **2004**, *9*, 1–18. <https://doi.org/10.1016/j.cocis.2004.05.004>.
- (35) Tadeo, X.; Pons, M.; Millet, O. Influence of the Hofmeister anions on protein stability as studied by thermal denaturation and chemical shift perturbation. *Biochem.* **2007**, *46*, 917–923. <https://doi.org/10.1021/bi0613426>.
- (36) Gibb, B. C. Hofmeister's Curse. *Nature Chemistry* **2019**, *11*, 963–965. <https://doi.org/10.1038/s41557-019-0355-1>.
- (37) Zhang, Y.; Cremer, P. S. Interactions between macromolecules and ions: the Hofmeister series. *Curr. Opin. Chem. Biol.* **2006**, *10*, 658–663. <https://doi.org/10.1016/j.cbpa.2006.09.020>.
- (38) Vlachy, N.; Jagoda-Cwiklik, B.; Vácha, R.; Touraud, D.; Jungwirth, P.; Kunz, W. Hofmeister series and specific interactions of charged headgroups with aqueous ions. *Adv. Colloid Interface Sci.* **2009**, *146*, 42–47. <https://doi.org/10.1016/j.cis.2008.09.010>.
- (39) Kunz, W. Specific ion effects in colloidal and biological systems. *Curr. Opin. Colloid Interface Sci.* **2010**, *15*, 34–39. <https://doi.org/10.1016/j.cocis.2009.11.008>.
- (40) Shao, Q.; Jiang, S. Influence of Charged Groups on the Properties of Zwitterionic Moieties: A Molecular Simulation Study. *J. Phys. Chem. B* **2014**, *118*, 7630–7637. <https://doi.org/10.1021/jp5027114>.
- (41) Sultana, N.; Ismail, K. Specific ion effects of chloride vis-à-vis acetate, propionate and butyrate counterions on the cetylpyridinium headgroup at the micelle–solution and air–solution interfaces. *J. Mol. Liq.* **2016**, *213*, 145–152. <https://doi.org/10.1016/j.molliq.2015.11.016>.
- (42) Mehringer, J.; Hofmann, E.; Touraud, D.; Koltzenburg, S.; Kellermeier, M.; Kunz, W. Salting-in and salting-out effects of short amphiphilic molecules: a balance between specific ion effects and hydrophobicity. *Phys. Chem. Chem. Phys.* **2021**, *23*, 1381–1391. <https://doi.org/10.1039/D0CP05491G>.
- (43) Li, R.; Xiong, Y. L. Sensitivity of oat protein solubility to changing ionic strength and pH. *J. Food Sci.* **2021**, *86*, 78–85. <https://doi.org/10.1111/1750-3841.15544>.

- (44) Hamada, H.; Arakawa, T.; Shiraki, K. Effect of additives on protein aggregation. *Curr. Pharm. Biotechnol.* **2009**, *10*, 400–407. <https://doi.org/10.2174/138920109788488941>.
- (45) Osen, R.; Toelstede, S.; Eisner, P.; Schweiggert-Weisz, U. Effect of high moisture extrusion cooking on protein–protein interactions of pea (*Pisum sativum* L.) protein isolates. *Int. J. Food Sci. Technol.* **2015**, *50*, 1390–1396. <https://doi.org/10.1111/ijfs.12783>.
- (46) Hatefi, Y.; Hanstein, W. G. Solubilization of particulate proteins and nonelectrolytes by chaotropic agents. *PNAS* **1969**, *62*, 1129–1136. <https://doi.org/10.1073/pnas.62.4.1129>.
- (47) McClements, D. J. Modulation of globular protein functionality by weakly interacting cosolvents. *Crit. Rev. Food Sci. Nutr.* **2002**, *42*, 417–471. <https://doi.org/10.1080/20024091054210>.
- (48) Mondal, S.; Moulik, S. Colloidal Dispersions of Lipids and Curcumin, and the Solubility and Degradation Kinetics of the Latter in Micellar Solution. *Soft Mater.* **2015**, *13*, 118–125. <https://doi.org/10.1080/1539445X.2015.1025981>.
- (49) Otzen, D. E.; Pedersen, J. N.; Østergaard Rasmussen, H.; Pedersen, J. S. How do surfactants unfold and refold proteins? *Adv. Colloid Interface Sci.* **2022**, *308*, 102754–102767. <https://doi.org/10.1016/j.cis.2022.102754>.
- (50) Liu, K.; Hsieh, F.-H. Protein–Protein Interactions during High-Moisture Extrusion for Fibrous Meat Analogues and Comparison of Protein Solubility Methods Using Different Solvent Systems. *J. Agric. Food Chem.* **2008**, *56*, 2681–2687. <https://doi.org/10.1021/jf073343q>.
- (51) Martin, A. H.; Nieuwland, M.; Jong, G. A. H. de. Characterization of Heat-Set Gels from RuBisCO in Comparison to Those from Other Proteins. *J. Agric. Food Chem.* **2014**, *62*, 10783–10791. <https://doi.org/10.1021/jf502905g>.
- (52) Smith, K.; Watson, A. W.; Lonnie, M.; Peeters, W. M.; Oonincx, D.; Tsoutsoura, N.; Simon-Miquel, G.; Szepe, K.; Cochetel, N.; Pearson, A. G.; *et al.* Meeting the global protein supply requirements of a growing and ageing population. *Eur. J. Nutr.* **2024**, *63*, 1425–1433. <https://doi.org/10.1007/s00394-024-03358-2>.
- (53) Burlingame, B.; Molledo, A.; Cafiero, C. Global protein sustainability and the United Nations, through to the 2030 agenda. *Frontiers in nutrition* **2024**, *11*, 1383898–1383906. <https://doi.org/10.3389/fnut.2024.1383898>.
- (54) Hoehnel, A.; Zannini, E.; Arendt, E. K. Targeted formulation of plant-based protein-foods: Supporting the food system’s transformation in the context of human health, environmental sustainability and consumer trends. *Trends Food Sci. Technol.* **2022**, *128*, 238–252. <https://doi.org/10.1016/j.tifs.2022.08.007>.
- (55) FAO 2024. FAOSTAT: Food Balances (2010-). <https://www.fao.org/faostat/en/#data/FBS> (accessed Dec 3, 2025).
- (56) The World Bank. World Bank Country and Lending Groups. <https://datahelpdesk.worldbank.org/knowledgebase/articles/906519-world-bank-country-and-lending-groups> (accessed Dec 3, 2025).
- (57) Steinfeld, H.; Gerber, P.; Wassenaar, T.; Castel, V.; Rosales, M.; Haan, C. de. *Livestock’s long shadow: environmental issues and options*; Rome, **2006**. ISBN 92-5-105571-8.

- (58) Good Food Institute. Modernizing meat production. <https://gfi.org/response-on-the-state-of-cultivated-meat-innovation/> (accessed Dec 3, 2025).
- (59) Sinke, P.; Swartz, E.; Sanctorem, H.; van der Giesen, C.; Odegard, I. Ex-ante life cycle assessment of commercial-scale cultivated meat production in 2030. *Int. J. Life Cycle Assess.* **2023**, *28*, 234–254. <https://doi.org/10.1007/s11367-022-02128-8>.
- (60) Ritchie, H.; Roser, M. Half of the world's habitable land is used for agriculture. <https://ourworldindata.org/global-land-for-agriculture> (accessed Dec 3, 2025).
- (61) Ritchie, H. If the world adopted a plant-based diet, we would reduce global agricultural land use from 4 to 1 billion hectares. <https://ourworldindata.org/land-use-diets> (accessed Dec 3, 2025).
- (62) Heinke, J.; Lannerstad, M.; Gerten, D.; Havlík, P.; Herrero, M.; Notenbaert, A. M. O.; Hoff, H.; Müller, C. Water Use in Global Livestock Production—Opportunities and Constraints for Increasing Water Productivity. *Water Resour. Res.* **2020**, *56*, 1–16. <https://doi.org/10.1029/2019WR026995>.
- (63) Garske, B.; Ekardt, F. Economic policy instruments for sustainable phosphorus management: taking into account climate and biodiversity targets. *Environ Sci Eur* **2021**, *33*, 1–20. <https://doi.org/10.1186/s12302-021-00499-7>.
- (64) Poore, J.; Nemecek, T. Reducing food's environmental impacts through producers and consumers. *Science* **2018**, *360*, 987–992. <https://doi.org/10.1126/science.aaq0216>.
- (65) United Nations. Sustainable Development Goals. <https://sdgs.un.org/goals> (accessed Dec 3, 2025).
- (66) United Nations. *Report of the Conf. of the Parties on its 21st Session, held in Paris from 30 November to 13 December 2015*, 2016. <https://unfccc.int/resource/docs/2015/cop21/eng/10.pdf> (accessed Dec 3, 2025).
- (67) Duluins, O.; Baret, P. V. A systematic review of the definitions, narratives and paths forwards for a protein transition in high-income countries. *Nature Food* **2024**, *5*, 28–36. <https://doi.org/10.1038/s43016-023-00906-7>.
- (68) Hadidi, M.; Tan, C.; Assadpour, E.; Kharazmi, M. S.; Jafari, S. M. Emerging plant proteins as nanocarriers of bioactive compounds. *J. Control Release* **2023**, *355*, 327–342. <https://doi.org/10.1016/j.jconrel.2023.01.069>.
- (69) Boukid, F.; Rosell, C. M.; Castellari, M. Pea protein ingredients: A mainstream ingredient to (re)formulate innovative foods and beverages. *Trends in Food Science & Technology* **2021**, *110*, 729–742. <https://doi.org/10.1016/j.tifs.2021.02.040>.
- (70) Wu, G.; Fanzo, J.; Miller, D. D.; Pingali, P.; Post, M.; Steiner, J. L.; Thalacker-Mercer, A. E. Production and supply of high-quality food protein for human consumption: sustainability, challenges, and innovations. *Ann. N. Y. Acad. Sci.* **2014**, *1321*, 1–19. <https://doi.org/10.1111/nyas.12500>.
- (71) Moughan, P. J. Population protein intakes and food sustainability indices: The metrics matter. *Glob. Food Secur.* **2021**, *29*, 100548–100556. <https://doi.org/10.1016/j.gfs.2021.100548>.

- (72) Aiking, H.; Boer, J. de. The next protein transition. *Trends in Food Science & Technology* **2020**, *105*, 515–522. <https://doi.org/10.1016/j.tifs.2018.07.008>.
- (73) Sá, A. G. A.; Moreno, Y. M. F.; Carciofi, B. A. M. Plant proteins as high-quality nutritional source for human diet. *Trends Food Sci. Technol.* **2020**, *97*, 170–184. <https://doi.org/10.1016/j.tifs.2020.01.011>.
- (74) Sun-Waterhouse, D.; Zhao, M.; Waterhouse, G. I. N. Protein Modification During Ingredient Preparation and Food Processing: Approaches to Improve Food Processability and Nutrition. *Food Bioprocess Technol.* **2014**, *7*, 1853–1893. <https://doi.org/10.1007/s11947-014-1326-6>.
- (75) Ge, J.; Sun, C.-X.; Corke, H.; Gul, K.; Gan, R.-Y.; Fang, Y. The health benefits, functional properties, modifications, and applications of pea (*Pisum sativum* L.) protein: Current status, challenges, and perspectives. *Compr Rev Food Sci Food Saf* **2020**, *19*, 1835–1876. <https://doi.org/10.1111/1541-4337.12573>.
- (76) Nesterenko, A.; Alric, I.; Silvestre, F.; Durrieu, V. Vegetable proteins in microencapsulation: A review of recent interventions and their effectiveness. *Ind. Crops Prod.* **2013**, *42*, 469–479. <https://doi.org/10.1016/j.indcrop.2012.06.035>.
- (77) Shanthakumar, P.; Klepacka, J.; Bains, A.; Chawla, P.; Dhull, S. B.; Najda, A. The Current Situation of Pea Protein and Its Application in the Food Industry. *Molecules* **2022**, *27*, 5354–5382. <https://doi.org/10.3390/molecules27165354>.
- (78) Gomes, A.; Sobral, P. J. Plant Protein-Based Delivery Systems: An Emerging Approach for Increasing the Efficacy of Lipophilic Bioactive Compounds. *Molecules* **2022**, *27*, 60–90. <https://doi.org/10.3390/molecules27010060>.
- (79) Akharume, F. U.; Aluko, R. E.; Adedeji, A. A. Modification of plant proteins for improved functionality: A review. *Compr Rev Food Sci Food Saf* **2021**, *20*, 198–224. <https://doi.org/10.1111/1541-4337.12688>.
- (80) Ihsan, A.; Ahmad, Z.; Zheng, J.; Bilal, M.; Muhammad Rizwan Abid, H.; Hu, A. New trends in functionalities and extraction of plant proteins in designing plant-based meat analogues: A critical review. *Food Biosci.* **2024**, *57*, 103476–103488. <https://doi.org/10.1016/j.fbio.2023.103476>.
- (81) Ma, K. K.; Greis, M.; Lu, J.; Nolden, A. A.; McClements, D. J.; Kinchla, A. J. Functional Performance of Plant Proteins. *Foods* **2022**, *11*, 594–617. <https://doi.org/10.3390/foods11040594>.
- (82) Singhal, A.; Karaca, A.; Tyler, R.; Nickerson, M. Pulse Proteins: From Processing to Structure-Function Relationships. In *Grain Legumes*; Goyal, A. K. (Ed.), **2016**, pp 55–79. <https://doi.org/10.5772/64020>.
- (83) Nasrabadi, M. N.; Sedaghat Doost, A.; Mezzenga, R. Modification approaches of plant-based proteins to improve their techno-functionality and use in food products. *Food Hydrocoll.* **2021**, *118*, 106789–106812. <https://doi.org/10.1016/j.foodhyd.2021.106789>.
- (84) Das, A.; Raychaudhuri, U.; Chakraborty, R. Cereal based functional food of Indian subcontinent: a review. *J. Food Sci. Technol.* **2012**, *49*, 665–672. <https://doi.org/10.1007/s13197-011-0474-1>.

- (85) Chandran, A. S.; Suri, S.; Choudhary, P. Sustainable plant protein: an up-to-date overview of sources, extraction techniques and utilization. *Sustainable Food Technol.* **2023**, *1*, 466–483. <https://doi.org/10.1039/D3FB00003F>.
- (86) Mudryj, A. N.; Yu, N.; Aukema, H. M. Nutritional and health benefits of pulses. *Appl. Physiol. Nutr. Metab.* **2014**, *39*, 1197–1204. <https://doi.org/10.1139/apnm-2013-0557>.
- (87) Kotecka-Majchrzak, K.; Sumara, A.; Fornal, E.; Montowska, M. Oilseed proteins – Properties and application as a food ingredient. *Trends Food Sci Tech* **2020**, *106*, 160–170. <https://doi.org/10.1016/j.tifs.2020.10.004>.
- (88) Kumar, M.; Tomar, M.; Potkule, J.; Verma, R.; Punia, S.; Mahapatra, A.; Belwal, T.; Dahuja, A.; Joshi, S.; Berwal, M. K.; *et al.* Advances in the plant protein extraction: Mechanism and recommendations. *Food Hydrocoll.* **2021**, *115*, 106595–106612. <https://doi.org/10.1016/j.foodhyd.2021.106595>.
- (89) Shewry, P. R.; Napier, J. A.; Tatham, A. S. Seed storage proteins: structures and biosynthesis. *Plant Cell* **1995**, *7*, 945–956. <https://doi.org/10.1105/tpc.7.7.945>.
- (90) Wakasa, Y.; Takaiwa, F. Seed Storage Proteins. In *Brenner's Encyclopedia of Genetics (Second Edition)*; Maloy, S., Hughes, K., Eds.; Academic Press: San Diego, **2013**; pp 346–348. <https://doi.org/10.1016/B978-0-12-374984-0.01378-4>.
- (91) Lu, Z. X.; He, J. F.; Zhang, Y. C.; Bing, D. J. Composition, physicochemical properties of pea protein and its application in functional foods. *Crit Rev Food Sci Nutr* **2020**, *60*, 2593–2605. <https://doi.org/10.1080/10408398.2019.1651248>.
- (92) FAO 2025. FAOSTAT: Crops and livestock products. <https://www.fao.org/faostat/en/#data/QCL> (accessed Dec 3, 2025).
- (93) Kumar, Y.; Basu, S.; Goswami, D.; Mridula, D.; Shivhare, U.; Vishwakarma, R. Anti-nutritional compounds in pulses: Implications and alleviation methods. *Legume Sci.* **2021**, *4*, 111–124. <https://doi.org/10.1002/leg3.111>.
- (94) Kong, C.-H.; Xuan, T. D.; Khanh, T. D.; Tran, H.-D.; Trung, N. T. Allelochemicals and Signaling Chemicals in Plants. *Molecules* **2019**, *24*, 2737–2756. <https://doi.org/10.3390/molecules24152737>.
- (95) Daba, S. D.; Morris, C. F. Pea proteins: Variation, composition, genetics, and functional properties. *Cereal Chem* **2022**, *99*, 8–20. <https://doi.org/10.1002/cche.10439>.
- (96) Nikolopoulou, D.; Grigorakis, K.; Stasini, M.; Alexis, M. N.; Iliadis, K. Differences in chemical composition of field pea (*Pisum sativum*) cultivars: Effects of cultivation area and year. *Food Chem.* **2007**, *103*, 847–852. <https://doi.org/10.1016/j.foodchem.2006.09.035>.
- (97) Barac, M.; Cabrilo, S.; Pesic, M.; Stanojevic, S.; Zilic, S.; Macej, O.; Ristic, N. Profile and functional properties of seed proteins from six pea (*Pisum sativum*) genotypes. *Int. J. Mol. Sci.* **2010**, *11*, 4973–4990. <https://doi.org/10.3390/ijms11124973>.
- (98) Gravel, A.; Doyen, A. Pulse Globulins 11S and 7S: Origins, Purification Methods, and Techno-functional Properties. *J. Agric. Food Chem.* **2023**, *71*, 2704–2717. <https://doi.org/10.1021/acs.jafc.2c07507>.

References

- (99) O'Kane, F. E.; Happe, R. P.; Vereijken, J. M.; Gruppen, H.; van Boekel, M. A. J. S. Characterization of pea vicilin. 1. Denoting convicilin as the alpha-subunit of the Pisum vicilin family. *J. Agric. Food Chem.* **2004**, *52*, 3141–3148. <https://doi.org/10.1021/jf035104i>.
- (100) Croy, R. R.; Gatehouse, J. A.; Tyler, M.; Boulter, D. The purification and characterization of a third storage protein (convicilin) from the seeds of pea (*Pisum sativum* L.). *Biochem. J.* **1980**, *191*, 509–516. <https://doi.org/10.1042/bj1910509>.
- (101) Boye, J.; Zare, F.; Pletch, A. Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International* **2010**, *43*, 414–431. <https://doi.org/10.1016/j.foodres.2009.09.003>.
- (102) Grasso, N.; Lynch, N. L.; Arendt, E. K.; O'Mahony, J. A. Chickpea protein ingredients: A review of composition, functionality, and applications. *Compr. Rev. Food Sci. Food Saf.* **2022**, *21*, 435–452. <https://doi.org/10.1111/1541-4337.12878>.
- (103) Summo, C.; Angelis, D. de; Ricciardi, L.; Caponio, F.; Lotti, C.; Pavan, S.; Pasqualone, A. Nutritional, physico-chemical and functional characterization of a global chickpea collection. *J. Food Compos. Anal.* **2019**, *84*, 103306–110315. <https://doi.org/10.1016/j.jfca.2019.103306>.
- (104) Serrano, M.; Carbas, B.; Castanho, A.; Soares, A.; Vaz Patto, M. C.; Brites, C. Characterisation of nutritional quality traits of a chickpea (*Cicer arietinum*) germplasm collection exploited in chickpea breeding in Europe. *Crop Pasture Sci.* **2017**, *68*, 1031–1040. <https://doi.org/10.1071/CP17129>.
- (105) Boukid, F. Chickpea (*Cicer arietinum* L.) protein as a prospective plant-based ingredient: a review. *Int. J. Food Sci. Technol.* **2021**, *56*, 5435–5444. <https://doi.org/10.1111/ijfs.15046>.
- (106) Hall, C.; Hillen, C.; Garden Robinson, J. Composition, Nutritional Value, and Health Benefits of Pulses. *Cereal Chem* **2017**, *94*, 11–31. <https://doi.org/10.1094/CCEM-03-16-0069-Fl>.
- (107) Papalamprou, E. M.; Doxastakis, G. I.; Kiosseoglou, V. Chickpea protein isolates obtained by wet extraction as emulsifying agents. *J. Sci. Food Agric.* **2010**, *90*, 304–313. <https://doi.org/10.1002/jsfa.3816>.
- (108) Wang, Y.; Li, K.; Bai, Y.; Li, B.; Xu, W. Effect of high intensity ultrasound on physicochemical, interfacial and gel properties of chickpea protein isolate. *LWT* **2020**, *129*, 109563–109572. <https://doi.org/10.1016/j.lwt.2020.109563>.
- (109) Biologie-Seite. Erbse. https://www.biologie-seite.de/Biologie/Datei:Illustration_Pisum_sativum0.jpg (accessed Dec 3, 2025).
- (110) Antiquariat Steffen Völkel. Foto von Cicer arietinum. https://www.zvab.com/servlet/BookDetailsPL?bi=31786737446&ref_=vhr_1_rvi_v2 (accessed Dec 3, 2025).
- (111) Pflanzen in Deutschland. Kartoffel - *Solanum tuberosum*. https://www.pflanzen-deutschland.de/Solanum_tuberosum.html (accessed Dec 3, 2025).

- (112) Bártová, V.; Bárta, J. Chemical composition and nutritional value of protein concentrates isolated from potato (*Solanum tuberosum* L.) fruit juice by precipitation with ethanol or ferric chloride. *Journal of Agricultural and Food Chemistry* **2009**, *57*, 9028–9034. <https://doi.org/10.1021/jf900897b>.
- (113) Ebert, S.; Gibis, M.; Terjung, N.; Weiss, J. Survey of aqueous solubility, appearance, and pH of plant protein powders from carbohydrate and vegetable oil production. *LWT* **2020**, *133*, 110078–110086. <https://doi.org/10.1016/j.lwt.2020.110078>.
- (114) Hussain, M.; Qayum, A.; Xiuxiu, Z.; Liu, L.; Hussain, K.; Yue, P.; Yue, S.; Y.F Koko, M.; Hussain, A.; Li, X. Potato protein: An emerging source of high quality and allergy free protein, and its possible future based products. *Food Res. Int.* **2021**, *148*, 110583–110594. <https://doi.org/10.1016/j.foodres.2021.110583>.
- (115) Waglay, A.; Karboune, S. Chapter 4 - Potato Proteins: Functional Food Ingredients. In *Advances in Potato Chemistry and Technology (Second Edition)*; Singh, J., Kaur, L., Eds.; Academic Press: San Diego, **2016**; pp 75–104. <https://doi.org/10.1016/B978-0-12-800002-1.00004-2>.
- (116) Pouvreau, L.; Alting, A.; Giuseppin, M.; Nieuwenhuijzen, N. H. Potato proteins. *Handbook of Food Proteins*, **2011**, 316–334. <https://doi.org/10.1533/9780857093639.316>.
- (117) Hu, C.; He, Y.; Zhang, W.; He, J. Potato proteins for technical applications: Nutrition, isolation, modification and functional properties - A review. *Innov. Food Sci. Emerg. Technol.* **2024**, *91*, 103533–103545. <https://doi.org/10.1016/j.ifset.2023.103533>.
- (118) Knorr, D.; Kohler, G. O.; Betschart, A. A. Potato protein concentrates: the influence of various methods of recovery upon yield, compositional and functional characteristics. *J. Food Process. and Preserv.* **1977**, *1*, 235–247. <https://doi.org/10.1111/j.1745-4549.1977.tb00326.x>.
- (119) Pęksa, A.; Miedzianka, J. Potato Industry By-Products as a Source of Protein with Beneficial Nutritional, Functional, Health-Promoting and Antimicrobial Properties. *Appl. Sci.* **2021**, *11*, 3497–3510. <https://doi.org/10.3390/app11083497>.
- (120) Racusen, D.; Weller, D. L. Molecular weight of patatin, a major potato tuber protein. *J. Food Biochem.* **1984**, *8*, 103–107. <https://doi.org/10.1111/j.1745-4514.1984.tb00318.x>.
- (121) Shewry, P. R. Tuber Storage Proteins. *Ann. Bot.* **2003**, *91*, 755–769. <https://doi.org/10.1093/aob/mcg084>.
- (122) van Koningsveld, G. A.; Gruppen, H.; Jongh, H. H. J. de; Wijngaards, G.; van Boekel, M. A. J. S.; Walstra, P.; Voragen, A. G. J. The solubility of potato proteins from industrial potato fruit juice as influenced by pH and various additives. *J. Sci. Food Agric.* **2002**, *82*, 134–142. <https://doi.org/10.1002/jsfa.1015>.
- (123) Tang, J.; Yao, D.; Xia, S.; Cheong, L.; Tu, M. Recent progress in plant-based proteins: From extraction and modification methods to applications in the food industry. *Food Chem.: X* **2024**, *23*, 101540–101554. <https://doi.org/10.1016/j.fochx.2024.101540>.
- (124) Ravindran, N.; Kumar Singh, S.; Singha, P. A comprehensive review on the recent trends in extractions, pretreatments and modifications of plant-based proteins. *Food Res. Int.* **2024**, *190*, 114575–114598. <https://doi.org/10.1016/j.foodres.2024.114575>.

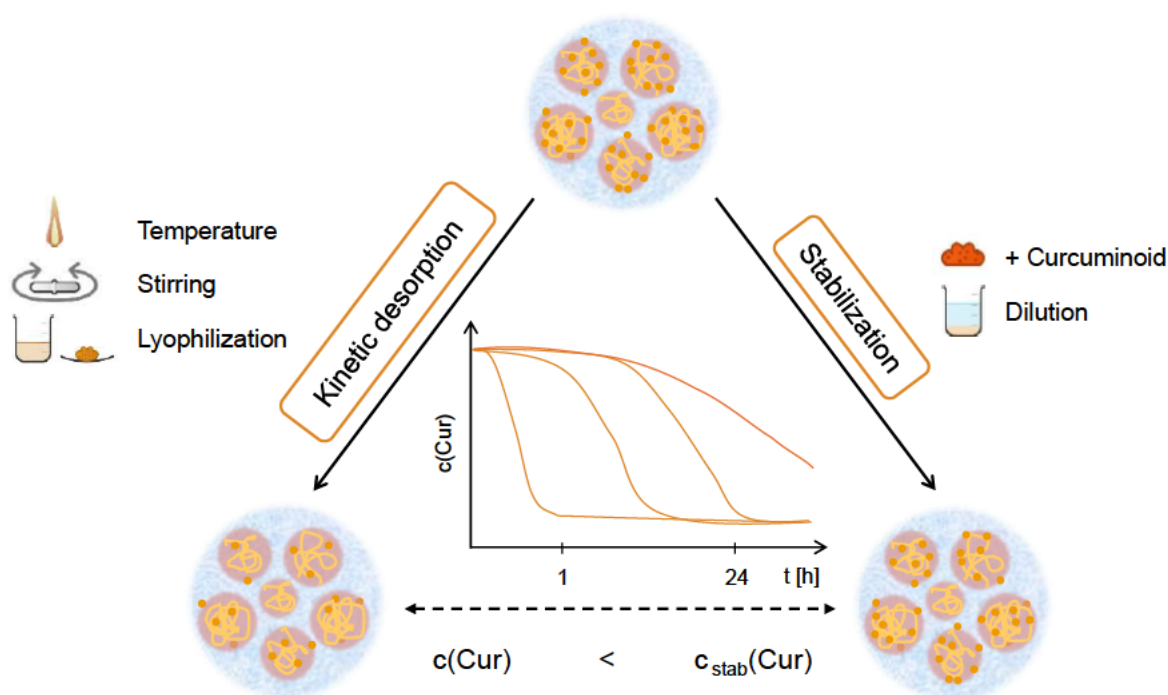
- (125) Pojić, M.; Mišan, A.; Brijesh, T. Eco-innovative technologies for extraction of proteins for human consumption from renewable protein sources of plant origin. *Trends Food Sci. Technol.* **2018**, *75*, 93–104. <https://doi.org/10.1016/j.tifs.2018.03.010>.
- (126) Tanger, C.; Engel, J.; Kulozik, U. Influence of extraction conditions on the conformational alteration of pea protein extracted from pea flour. *Food Hydrocoll.* **2020**, *107*, 105949–105964. <https://doi.org/10.1016/j.foodhyd.2020.105949>.
- (127) Hadidi, M.; Aghababaei, F.; McClements, D. J. Enhanced alkaline extraction techniques for isolating and modifying plant-based proteins. *Food Hydrocoll.* **2023**, *145*, 109132–109149. <https://doi.org/10.1016/j.foodhyd.2023.109132>.
- (128) Giuseppin, M. L. F.; Laus, M. C.; Schipper, J. Potato protein isolates. WO 2014/011042 A1, **2014**.
- (129) Reinshagen, P. Cold recovery of potato proteins, a wonderful innovation. <https://www.biobasedpress.eu/2013/03/cold-recovery-of-potato-proteins-a-wonderful-innovation/> (accessed Dec 4, 2025).
- (130) Zhang, S. Fabrication of novel biomaterials through molecular self-assembly. *Nat. Biotechnol.* **2003**, *21*, 1171–1178. <https://doi.org/10.1038/nbt874>.
- (131) Hewage, A.; Olatunde, O. O.; Nimalaratne, C.; Malalgoda, M.; Aluko, R. E.; Bandara, N. Novel Extraction technologies for developing plant protein ingredients with improved functionality. *Trends Food Sci. Technol.* **2022**, *129*, 492–511. <https://doi.org/10.1016/j.tifs.2022.10.016>.
- (132) Lima, R. R.; Stephani, R.; Perrone, Í. T.; Carvalho, A. F. de. Plant-based proteins: A review of factors modifying the protein structure and affecting emulsifying properties. *Food Chemistry Advances* **2023**, *3*, 100397–100411. <https://doi.org/10.1016/j.focha.2023.100397>.
- (133) Ye, S.-J.; Park, H.-J.; Baik, M.-Y. Modification of plant proteins as alternatives to animal proteins: a review. *Food Sci. Biotechnol.* **2025**, *34*, 349–363. <https://doi.org/10.1007/s10068-024-01705-x>.
- (134) Aboud, S. A.; Altemimi, A. B.; Al-Hilphy, A. R. S.; Yi-Chen, L.; Cacciola, F. A Comprehensive Review on Infrared Heating Applications in Food Processing. *Molecules* **2019**, *24*, 4125–4145. <https://doi.org/10.3390/molecules24224125>.
- (135) Zhao, Y.; Flugstad, B. E.; Kolbe, E.; Park, J. W.; Wells, J. H. Using capacitive (radio frequency) dielectric heating in food processing and preservation – a review. *J. Food Process Eng.* **2000**, *23*, 25–55. <https://doi.org/10.1111/j.1745-4530.2000.tb00502.x>.
- (136) Pismag, R. Y.; Polo, M. P.; Hoyos, J. L.; Bravo, J. E.; Roa, D. F. Effect of extrusion cooking on the chemical and nutritional properties of instant flours: a review. *F1000Research* **2023**, *12*, 1356–1381. <https://doi.org/10.12688/f1000research.140748.2>.
- (137) O’Sullivan, J.; Murray, B.; Flynn, C.; Norton, I. The effect of ultrasound treatment on the structural, physical and emulsifying properties of animal and vegetable proteins. *Food Hydrocoll.* **2016**, *53*, 141–154. <https://doi.org/10.1016/j.foodhyd.2015.02.009>.
- (138) Margulis, M. A.; Margulis, I. M. Calorimetric method for measurement of acoustic power absorbed in a volume of a liquid. *Ultrason. Sonochem.* **2003**, *10*, 343–345. [https://doi.org/10.1016/s1350-4177\(03\)00100-7](https://doi.org/10.1016/s1350-4177(03)00100-7).

- (139) Hamada, J. S. Deamidation of food proteins to improve functionality. *Crit Rev Food Sci Nutr* **1994**, *34*, 283–292. <https://doi.org/10.1080/10408399409527664>.
- (140) Lin, D.; Sun, L.-C.; Chen, Y.-L.; Liu, G.-M.; Miao, S.; Cao, M.-J. Peptide/protein hydrolysate and their derivatives: Their role as emulsifying agents for enhancement of physical and oxidative stability of emulsions. *Trends Food Sci. Technol.* **2022**, *129*, 11–24. <https://doi.org/10.1016/j.tifs.2022.08.012>.
- (141) Stanbury, P. F.; Whitaker, A.; Hall, S. J. Chapter 1 - An introduction to fermentation processes. In *Principles of Fermentation Technology (Third Edition)*; Stanbury, P. F., Whitaker, A., Hall, S. J., Eds.; Butterworth-Heinemann: Oxford, **2017**; pp 1–20. <https://doi.org/10.1016/B978-0-08-099953-1.00001-6>.
- (142) Pecora, R. Dynamic Light Scattering Measurement of Nanometer Particles in Liquids. *J. Nanopart. Res.* **2000**, *2*, 123–131. <https://doi.org/10.1023/A:1010067107182>.
- (143) Smith, A. K.; Rackis, J. J.; Isnardi, P.; Carter, J. L.; Krober, O. A. Nitrogen solubility index, isolated protein yield, and whey nitrogen content of several soybean strains. *Cereal Chem* **1966**, 261–269.
- (144) Mariotti, F.; Tomé, D.; Mirand, P. P. Converting nitrogen into protein—beyond 6.25 and Jones’ factors. *Crit Rev Food Sci Nutr* **2008**, *48*, 177–184. <https://doi.org/10.1080/10408390701279749>.
- (145) Sun, H.; Sun, J.; Dou, N.; Li, J.; Hussain, M. A.; Ma, J.; Hou, J. Characterization and comparison of structure, thermal and functional characteristics of various commercial pea proteins. *Food Biosci.* **2023**, *53*, 102740–102751. <https://doi.org/10.1016/j.fbio.2023.102740>.
- (146) Shand, P. J.; Ya, H.; Pietrasik, Z.; Wanasundara, P. Physicochemical and textural properties of heat-induced pea protein isolate gels. *Food Chem.* **2007**, *102*, 1119–1130. <https://doi.org/10.1016/j.foodchem.2006.06.060>.
- (147) Romero, A.; Beaumal, V.; David-Briand, E.; Cordobés, F.; Guerrero, A.; Anton, M. Interfacial and Oil/Water Emulsions Characterization of Potato Protein Isolates. *J. Agric. Food Chem.* **2011**, *59*, 9466–9474. <https://doi.org/10.1021/jf2019853>.
- (148) Andlinger, D. J.; Röscheisen, P.; Hengst, C.; Kulozik, U. Influence of pH, Temperature and Protease Inhibitors on Kinetics and Mechanism of Thermally Induced Aggregation of Potato Proteins. *Foods* **2021**, *10*, 796–815. <https://doi.org/10.3390/foods10040796>.
- (149) Ricci, L.; Umiltà, E.; Righetti, M. C.; Messina, T.; Zurlini, C.; Montanari, A.; Bronco, S.; Bertoldo, M. On the thermal behavior of protein isolated from different legumes investigated by DSC and TGA. *J. Sci. Food Agric.* **2018**, *98*, 5368–5377. <https://doi.org/10.1002/jsfa.9078>.
- (150) Morales-Quintana, L.; Tapia-Valdebenito, D.; Castro, R. I.; Rabert, C.; Larama, G.; Gutiérrez, A.; Ramos, P. Characterization of the Cell Wall Component through Thermogravimetric Analysis and Its Relationship with an Expansin-like Protein in *Deschampsia antarctica*. *Int. J. Mol. Sci.* **2022**, *23*, 5741–5755. <https://doi.org/10.3390/ijms23105741>.
- (151) Mession, J.-L.; Sok, N.; Assifaoui, A.; Saurel, R. Thermal Denaturation of Pea Globulins (*Pisum sativum* L.)—Molecular Interactions Leading to Heat-Induced Protein Aggregation. *J. Agric. Food Chem.* **2013**, *61*, 1196–1204. <https://doi.org/10.1021/jf303739n>.

- (152) Arntfield, S. D.; Murray, E. D. The Influence of Processing Parameters on Food Protein Functionality I. Differential Scanning Calorimetry as an Indicator of Protein Denaturation. *Can. Inst. Food Sci. Technol. J.* **1981**, *14*, 289–294. [https://doi.org/10.1016/S0315-5463\(81\)72929-8](https://doi.org/10.1016/S0315-5463(81)72929-8).
- (153) van Koningsveld, G. A.; Gruppen, H.; Jongh, H. H. de; Wijngaards, G.; van Boekel, M. A.; Walstra, P.; Voragen, A. G. Effects of pH and heat treatments on the structure and solubility of potato proteins in different preparations. *J. Agric. Food Chem.* **2001**, *49*, 4889–4897. <https://doi.org/10.1021/jf010340j>.
- (154) Schmidt, V.; Giacomelli, C.; Soldi, V. Thermal stability of films formed by soy protein isolate–sodium dodecyl sulfate. *Polym. Degrad. Stab.* **2005**, *87*, 25–31. <https://doi.org/10.1016/j.polymdegradstab.2004.07.003>.
- (155) Chemat, A.; Touraud, D.; Müller, R.; Kunz, W.; Fabiano-Tixier, A.-S. Valorization of Coffee Silverskin Using Extraction Cycles and Water as a Solvent: Design of Process. *Molecules* **2024**, *29*, 1318–1332. <https://doi.org/10.3390/molecules29061318>.
- (156) Zhao, R.; Fu, W.; Li, D.; Dong, C.; Bao, Z.; Wang, C. Structure and functionality of whey protein, pea protein, and mixed whey and pea proteins treated by pH shift or high-intensity ultrasound. *J. Dairy Sci.* **2024**, *107*, 726–741. <https://doi.org/10.3168/jds.2023-23742>.
- (157) Tang, C.-H. Assembly of food proteins for nano- encapsulation and delivery of nutraceuticals (a mini-review). *Food Hydrocoll.* **2021**, *117*, 106710–106721. <https://doi.org/10.1016/j.foodhyd.2021.106710>.
- (158) Wang, W. Protein aggregation and its inhibition in biopharmaceutics. *Int. J. Pharm.* **2005**, *289*, 1–30. <https://doi.org/10.1016/j.ijpharm.2004.11.014>.
- (159) Hadidi, M.; Boostani, S.; Jafari, S. Pea proteins as emerging biopolymers for the emulsification and encapsulation of food bioactives. *Food Hydrocoll.* **2022**, *126*, 107474–107490. <https://doi.org/10.1016/j.foodhyd.2021.107474>.
- (160) Sharif, H. R.; Williams, P. A.; Sharif, M. K.; Abbas, S.; Majeed, H.; Masamba, K. G.; Safdar, W.; Zhong, F. Current progress in the utilization of native and modified legume proteins as emulsifiers and encapsulants – A review. *Food Hydrocoll.* **2018**, *76*, 2–16. <https://doi.org/10.1016/j.foodhyd.2017.01.002>.
- (161) Chao, D.; Aluko, R. Modification of the structural, emulsifying, and foaming properties of an isolated pea protein by thermal pretreatment. *CyTA - Journal of Food* **2018**, *16*, 357–366. <https://doi.org/10.1080/19476337.2017.1406536>.
- (162) Ninham, B. W. *Increasing the solubility of proteins in aqueous solutions by reducing hydrophobic air bubbles in the solution*. Dialogue with Kunz, W., **2022**.
- (163) Lorsch, J., Ed. *Methods in Enzymology: Laboratory Methods in Enzymology: Protein Part C*; Academic Press, **2014**. ISBN 9780124201194.
- (164) Fusina, A.; Degot, P.; Touraud, D.; Kunz, W.; Nardello-Rataj, V. Enhancement of water solubilization of quercetin by meglumine and application of the solubilization concept to a similar system. *J. Mol. Liq.* **2022**, *368*, 120756–120766. <https://doi.org/10.1016/j.molliq.2022.120756>.

- (165) Li, H.; Ma, L.; Li, X.; Cui, X.; Yang, W.; Shen, S.; Chen, M. A simple and effective method to improve bioavailability of glimepiride by utilizing hydrotrophy technique. *Eur. J. Pharm. Sci.* **2015**, *77*, 154–160. <https://doi.org/10.1016/j.ejps.2015.06.016>.
- (166) Degot, P.; Funkner, D.; Huber, V.; Köglmaier, M.; Touraud, D.; Kunz, W. Extraction of curcumin from *Curcuma longa* using meglumine and pyroglutamic acid, respectively, as solubilizer and hydrotrope. *Journal of Molecular Liquids* **2021**, *334*, 116478–116487. <https://doi.org/10.1016/j.molliq.2021.116478>.
- (167) Leung, M. H. M.; Colangelo, H.; Kee, T. W. Encapsulation of Curcumin in Cationic Micelles Suppresses Alkaline Hydrolysis. *Langmuir* **2008**, *24*, 5672–5675. <https://doi.org/10.1021/la800780w>.
- (168) Schenk, J. J.; Becklund, L. E.; Carey, S. J.; Fabre, P. P. What is the “modified” CTAB protocol? Characterizing modifications to the CTAB DNA extraction protocol. *Appl. Plant Sci.* **2023**, *11*, 11517–11528. <https://doi.org/10.1002/aps3.11517>.
- (169) Johnston, C. S.; Steinberg, F. M.; Rucker, R. Ascorbic Acid. *Handbook of Vitamins* **2013**, 489–520. <https://doi.org/10.1201/b15413-15>.
- (170) Nafchi, A. M.; Tabatabaei, R. H.; Pashania, B.; Rajabi, H. Z.; Karim, A. A. Effects of ascorbic acid and sugars on solubility, thermal, and mechanical properties of egg white protein gels. *Int. J. Biol. Macromol.* **2013**, *62*, 397–404. <https://doi.org/10.1016/j.ijbiomac.2013.09.050>.
- (171) Nemet, I.; Monnier, V. M. Vitamin C degradation products and pathways in the human lens. *J. Biol. Chem.* **2011**, *286*, 37128–37136. <https://doi.org/10.1074/jbc.M111.245100>.
- (172) Seibles, T. S. Studies on potato proteins. *Am. Potato J.* **1979**, *56*, 415–425. <https://doi.org/10.1007/BF02854059>.
- (173) Jiang, S.; Ding, J.; Andrade, J.; Rababah, T. M.; Almajwal, A.; Abulmeaty, M. M.; Feng, H. Modifying the physicochemical properties of pea protein by pH-shifting and ultrasound combined treatments. *Ultrason. Sonochem.* **2017**, *38*, 835–842. <https://doi.org/10.1016/j.ultsonch.2017.03.046>.
- (174) Lee, H.; Yildiz, G.; dos Santos, L. C.; Jiang, S.; Andrade, J. E.; Engeseth, N. J.; Feng, H. Soy protein nano-aggregates with improved functional properties prepared by sequential pH treatment and ultrasonication. *Food Hydrocoll.* **2016**, *55*, 200–209. <https://doi.org/10.1016/j.foodhyd.2015.11.022>.
- (175) Guo, Q.; Su, J.; Shu, X.; Yuan, F.; Mao, L.; Liu, J.; Gao, Y. Production and characterization of pea protein isolate-pectin complexes for delivery of curcumin: Effect of esterified degree of pectin. *Food Hydrocoll.* **2020**, *105*, 105777–105788. <https://doi.org/10.1016/j.foodhyd.2020.105777>.
- (176) Abioye, A.; Abioye, V.; Ade-Omowaye, B.; Adedeji, A. Kinetic modeling of ascorbic acid loss in baobab drink at pasteurization and storage temperatures. *IOSR-JESTFT* **2013**, *7*, 17–23. <https://doi.org/10.9790/2402-0721723>.
- (177) Barth, A. Infrared spectroscopy of proteins. *Biochimica et biophysica acta* **2007**, *1767*, 1073–1101. <https://doi.org/10.1016/j.bbabbio.2007.06.004>.

Chapter 3 Kinetic Solubilization of Curcumin by Pea Protein Isolate



Significant parts of this chapter have been submitted for publication (Rohr, L.; Stefan, N.; Bruckmann, A.; Touraud, D.; Dürkop, A.; Sprangers, R.; Kunz, W. Mechanistic insights into the preparation and stabilisation of supersaturated pea protein isolate-curcumin nanoparticles prepared by a pH-driven method. Food Chem. 2026. *Under review.*). Nadine Stefan performed SDS-PAGE and SEC on my behalf. Dr. Astrid Bruckmann performed and evaluated MS. Prof. Dr. Axel Dürkop provided access to CD. Dr. Martin Schmid performed XRD on my behalf. Sabrina Zölch provided instructions and chemicals for preparing the simulated intestinal fluid (SIF). Jennifer Winter, Katharina Winter, Sandra Nguyen, Fabian Gliese, Anna Mändl, and Anna Selmeier provided support in performing PPICur stability and stabilization experiments.

3.1. Abstract

Curcumin (Cur), a polyphenol found in turmeric, has been extensively studied for its coloring and therapeutic properties. However, its application is mainly limited by its low water solubility. The water solubility of curcumin was enhanced by forming nanoparticles from a commercial pea protein isolate and curcumin, using a widely accepted pH-driven method. The process exhibited a significant supersaturation of curcumin – 96% of the initially solubilized curcumin precipitated within 24 hours under constant stirring, resulting in a stable solution of pea protein isolate-curcumin nanoparticles (PPICur). The supersaturated and stable mass concentrations were 1.39 ± 0.093 mg/mL and 0.0528 ± 0.0088 mg/mL, respectively, compared to a curcumin solubility in water of only a few $\mu\text{g/mL}$. The following chapter evaluates the stability and factors affecting the kinetic adsorption and desorption of curcumin to and from the plant protein nanoparticles by comparing the supersaturated and stable states. Along with current literature, a schematic mechanism covering all stages from nanoparticle preparation to curcumin precipitation is proposed. Two hypotheses are suggested to explain the observed phenomenon: the aggregation of curcumin molecules, closely packed on the nanoparticle surface, or the isomerization and subsequent aggregation of curcumin. Precipitation kinetics are significantly affected by temperature, stirring, and lyophilization, and can be slowed by adding bisdemethoxycurcumin and dilution. Understanding the relationship between the preparation method, its effect on nanoparticle structure, and the kinetics of curcumin adsorption and desorption bridges a gap in existing literature. By presenting mechanism-based stabilization strategies, this study contributes to clarifying the potential of plant-protein-based delivery systems and enhances the applicability of Cur in the food and pharmaceutical industries.

3.2. Fundamental Information

3.2.1. Curcumin

Curcumin (Cur) is a compound naturally found in the rhizomes of *Curcuma longa* L., commonly known as turmeric (see botanical illustration of the plant in Figure 3.1 left). Turmeric belongs to the *Zingiberaceae* family and is mainly cultivated in Asia, with India as the world's leading producer. The dried and ground powder of the rhizomes has been used for centuries as a spice, colorant, and therapeutic agent in traditional Chinese medicine and Ayurveda¹⁻⁴. While its aromatic properties are attributed to volatile essential oils, its vibrant orange color and antioxidant activity come from the group of curcuminoids^{3,5}. Curcuminoids are a group of polyphenols that constitute 1-6% (w/w) of the dry rhizome, depending on the cultivation region⁶⁻⁸. The main forms differ only in the number of methoxy groups, with Cur being the most abundant, accounting for about 77%, followed by demethoxycurcumin at 17-18% and bisdemethoxycurcumin at 3-5%^{5,9,10}. The molecular structures are depicted in Figure 3.1 on the right.

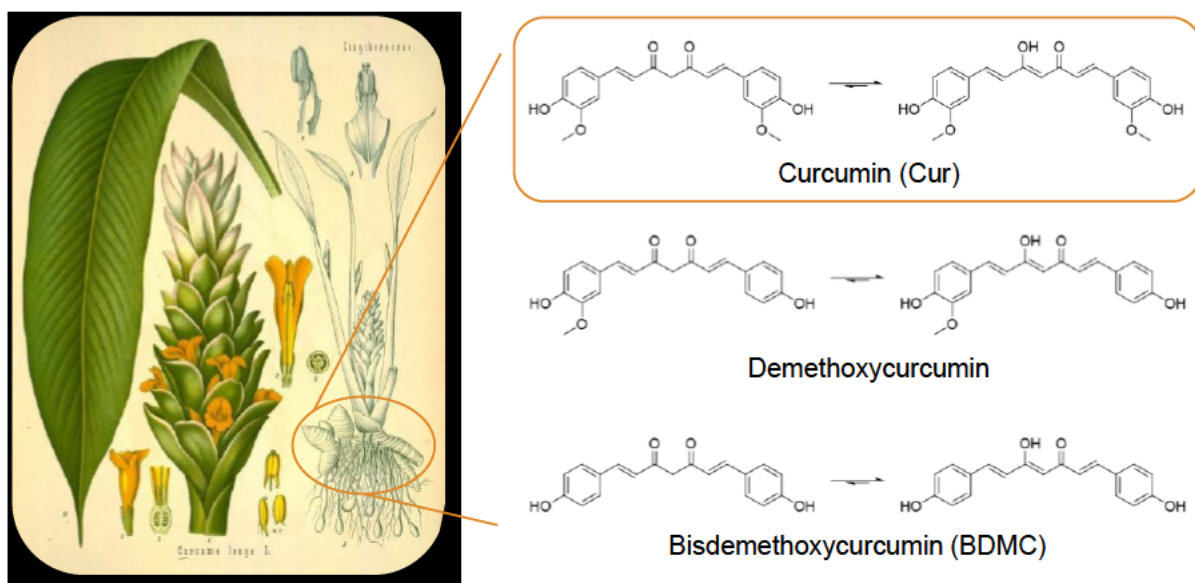


Figure 3.1: Botanical illustration of *Curcuma Longa* L.¹¹ (graphic of public domain) and molecular structures of curcuminoids, including curcumin (Cur), demethoxycurcumin, and bisdemethoxycurcumin.

On an industrial scale, curcuminoids are extracted using organic solvent methods such as Soxhlet extraction and maceration. Efforts have been made to reduce the use of organic solvents or to employ more environmentally friendly solvents in the extraction process. Innovative techniques, such as ultrasound-assisted extraction, enzyme-assisted extraction, supercritical fluid extraction, or green solvent extraction, have been developed to achieve this goal, although they are not yet used on an industrial scale^{8,12}. Mixtures of ethanol and water have been introduced as less harmful solvent systems, but they produce lower extraction yields. Degot *et al.* developed an edible, surfactant-free

microemulsion consisting of ethanol, triacetin, and water, which increased extraction yield by 53% compared to ethanol-water mixtures^{13,14}. Huber *et al.* replaced the water phase with a natural deep eutectic solvent, achieving nearly complete extraction of the curcuminoids, with Soxhlet extraction values serving as a reference^{15,16}. While extraction from natural sources provides a biologically authentic curcuminoid mixture, chemical synthesis allows for standardized, pure, and reproducible production of isolated curcuminoids^{10,12}. Experimentally, this offers the opportunity to examine different perspectives: while *in vivo* studies and clinical trials favor the biologically authentic mixtures, more fundamental studies tend to use synthetic Cur to create reproducible experimental conditions and to focus on the most abundant curcuminoid^{3,17}. Since the latter also applies to the chapter discussed here, only Cur will be covered in the following.

The molecular structure of Cur contains various chemical functionalities. Its bis- α , β -unsaturated β -diketone structure allows it to undergo keto-enol tautomerism. It can act as both a hydrogen bond donor and acceptor through the β -dicarbonyl moiety and hydroxyl groups, and it functions as a Michael acceptor. This molecular configuration makes Cur highly sensitive to environmental factors such as pH and light¹⁸. The enol group and the phenolic hydroxy groups are weakly acidic with pK_a values of 7.7-8.5, 8.5-10.4, and 9.5-10.7, respectively^{19,20}. In water and buffer systems at neutral pH, the reported solubility of the uncharged form ranges from 11 ng/mL to 10 μ g/mL, or 30 nM^{4,17}. Its low solubility results not only from its hydrophobic nature but also from π - π -stacking of its planar aromatic structure. Upon deprotonation, the solubility increases significantly, and the color shifts from yellow to orange to brown^{19,21,22}. In both protonated and deprotonated states, Cur degrades within minutes. The primary degradation products, depending on pH, light exposure, or autoxidation conditions, include vanillin, vanillic acid, feruloyl methane, ferulic acid, feruloyl aldehyde, hydroxybenzoic acid, and bicyclopentadione^{3,4,17}.

Due to the extended π -electron system in the enol form, its designation as “generally recognized as safe” (GRAS) by the US Food & Drug Administration (FDA)²³, its historical use in traditional medicine, and some promising results from *in vitro* studies, Cur is not only studied as a substitute for potentially harmful food colorings²⁴ but research also extends into the medical field. There, the scope ranges from Cur’s use as a nutraceutical⁴, i.e., a food component with health-promoting benefits²⁵, to its use as an active agent against diabetes²⁶, Alzheimer’s disease²⁷, and cancer^{4,17,28}. On the one hand, there is enormous interest in Cur as a potential treatment for serious diseases; on the other hand, there is harsh criticism of Cur as an improbable or invalid metabolic panacea (IMP)^{17,29}. Nelson *et al.* published a comprehensive overview of the criticisms of panacea-related research in that field in 2017¹⁷. Although the classification and investigation of the health-related properties of curcumin are far beyond the scope of this thesis, they will be briefly discussed below, since most research resources focus on this area.

The mode of action of a potential drug is described by two complementary fields, pharmacokinetics and pharmacodynamics. Pharmacokinetics relates to how a drug behaves in the body after administration, meaning what the body does with the drug. Pharmacodynamics, on the other hand, outlines the interaction between drug concentration and pharmacological effect, meaning what the drug does to the body^{30,31}. Pharmacokinetic criteria are summarized by the acronym ADME, which stands for absorption, distribution, metabolism, and excretion³². The ADME properties of Cur are described as follows: studies and clinical trials were conducted with oral doses of Cur ranging from 8 to 12 g/day. Despite this high dose, absorption, indicated by detectable blood serum concentrations, was minimal^{4,33,34}. The distribution of Cur to specific tissues or organs has been reported controversially, but mainly limited. Possible reasons include poor initial absorption, lack of standardized evaluation procedures, and compound degradation immediately after application. If the first two criteria—absorption and distribution – could be improved, the rapid metabolism of Cur in the body alters its molecular structure by reducing double bonds (phase I metabolism) and conjugating Cur and its reduced forms (phase II metabolism). Due to low absorption and distribution, along with rapid metabolism, Cur was mainly excreted unchanged in the feces. Rarely, metabolites were found in the urine, or it was also reported that Cur degrades and becomes undetectable¹⁷. Due to its low solubility and stability in biological systems, Cur has poor pharmacokinetic properties and thus low bioavailability³⁰. To evaluate pharmacodynamics effectively, the pharmacokinetic properties must first be optimized. Many parameters are currently circulating in the literature, such as differing bioavailability values of various formulations, varying effects of these formulations on Cur metabolism, and discrepancies between turmeric and Cur, which make comparison and accurate assessment of pharmacodynamics challenging.

Besides its poor ADME properties, Cur is also classified as a PAINS (pan-assay interference compound). Compounds with this label are known to generate false-positive results in various assay formats. Common assay interferences include fluorescence interference, metal chelation, covalent binding to proteins, redox reactivity, aggregation, membrane disruption, and degradation. Therefore, it is recommended to perform multiple control tests of the obtained results. Other highly researched compounds classified as PAINS include resveratrol, quercetin, capsaicin, and epigallocatechin gallate^{17,35}. Nelson *et al.* strongly criticize the lack of clinical studies to justify the significant research and financial costs. They further summarize that poor Cur absorption might be the limiting factor and that health-beneficial effects of Cur could be achieved through absorption-independent mechanisms, such as influencing the gut microbiota¹⁷. Hedge *et al.* published a comprehensive review in 2023 of clinical trials of Cur formulations with increased bioavailability. Since the publication of Nelson *et al.*, a promising number of randomized, placebo-controlled, double-blind clinical trials have reported

positive effects of Cur on type 2 diabetes, osteoarthritis, cognitive health in the elderly, and premenstrual syndrome³⁶.

Handling of Cur remains challenging (see overview in Figure 3.2), as does accessing its highly praised health benefits to a level that proves a positive impact on human health. In summary, whether used as a nutraceutical, a drug, or a food colorant, improving the aqueous solubility and stability of Cur is essential to accessing most of its benefits^{4,36,37}. This can be achieved through various solubilization systems, which will be discussed in the next section.

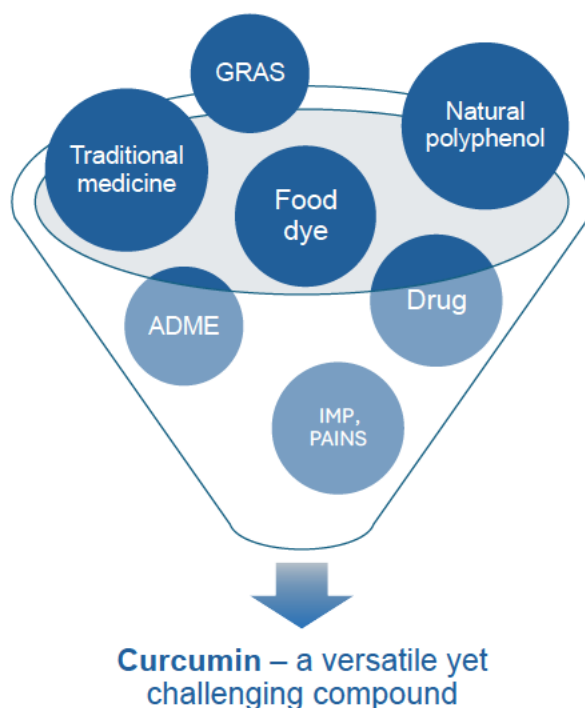


Figure 3.2: Overview of factors to consider when working with Cur and its potential applications.

3.2.2. Solubilization Strategies of Curcumin in Aqueous Systems

3.2.2.1. Overview of Molecular and Colloidal Solubilization Systems

To enhance the solubility and stability of Cur in aqueous systems, extensive research has been conducted on molecular and colloidal solubilization mechanisms^{19,38–40}. In Table 3.1 different solubilization systems are summarized, including the reagents used and their solubilization performance. A wide range of concentrations is reported for each category, varying from a few µg/mL to two-digit mg/mL. The highest solubilities were reported for emulsion-based systems. Selecting a suitable carrier system requires careful adaptation to the matrix, as well as consideration of the corresponding functional, technological, and regulatory requirements. Joye *et al.* provided a detailed overview of the advantages and disadvantages of selected carrier systems for food applications⁴¹. Enhancing bioavailability is often the primary goal after increasing solubility. Consequently, evaluating bioavailability significantly impacts the selection of carrier systems for most applications, except for coloring purposes^{39–41}. Since this chapter focuses only on solubilization performance, bioavailability evaluations are deliberately excluded.

Delivery systems based on proteins have attracted considerable interest from both the food and pharmaceutical sectors due to their ability to form diverse nanoarchitectures. These depend on the processing conditions of the proteins and their interactions with the target compound, making proteins a versatile and sustainable platform for formulating lipophilic active ingredients in aqueous media^{42–45}. There is a growing trend toward replacing animal proteins with plant-based alternatives. The reasons for this shift and the benefits of plant proteins are discussed in detail in Chapter 2 and are therefore not repeated here. In particular, complexes of plant proteins and Cur have attracted attention, as they offer not only good solubilization and stabilization properties but also significant biological activity along with a straightforward, solvent-free preparation, biodegradability, and biocompatibility^{46,47}. This method of Cur solubilization should be explored in more detail in the following section.

Table 3.1: Summary of examples for molecular and colloidal solubilization of Cur in aqueous systems. The reagents used and Cur concentrations obtained are included.

Carrier/Solubilization system	Reagent(s)	Comments	c(Cur)	Source
Micellar solubilization	TWEEN 60	c(TWEEN) = 1 mM	10 μ M	48
	TWEEN 20	c(TWEEN) = 1-10 x cmc	10 μ M	49
	Sodium caseinate (NaCas)	c(NaCas) = 0.02 mg/mL	137 μ g/mL	50
	Soluplus	c(Soluplus) = 10% (w/v)	50 $\pm$ 10 μ M	51
	Rhamnolipid (Rha)	c(Rha) = 8 mg/mL	0.8 mg/mL	52
	Sophorolipid (Sop)	c(Sop) = 6 mg/mL	0.9 mg/mL	53
	Hydrolysed pea protein	c(Protein) = 0.5% (w/w)	0.16 mg/mL	54
	Octenylsuccinated corn dextrin (OSCD)	c(OSCD) = 20g/L	0.5 mg/mL	55
	Dextran- deoxycholic acid (Dex-DeA)	c(Dex-DeA) = 5 mg/mL	0.75 mg/mL	56
	Hydroxypropylated starch	c(Starch) = 2.5 mg/mL	130.56 $\pm$ 9.75 μ g/mL	57
Cyclodextrin complexation	Hydrophobically modified starch	c(Starch) = 1% (w/v)	18.4 μ g/mL	58
	Randomly methylated β -cyclodextrin (CD)	c(CD) = 11% (w/v)	290 μ g/mL	59
	β -Cyclodextrin	c(CD) = 4.8 mg/mL	1.2 mg/mL	60
	Hydroxypropyl- γ -cyclodextrin	c(CD) = 10% (w/v)	5.35 mM	61
Salt formation	Arginine	c(Arg) = c(Cur) in mol/L, pH 8.7	0.88 mg/mL	62

Table 3.1 continued.

Carrier/Solubilization system	Reagent(s)	Comments	c(Cur)	Source
Emulsion systems	Silica, water, canola oil	Pickering emulsion	200 µg/g	63
	Whey protein, water/PBS, medium-chain triglyceride oil	Pickering emulsion	474 ± 29.4 µg/mL	64
	Quinoa starch granules, PBS, Miglyol 812	Pickering emulsion	17.5 µg/mL	65
	Pea protein nanoparticles, PBS, corn oil	Pickering emulsion	0.87 mg/g	66
	TWEEN 80, vitamin E, water, PEG-400	Nanoemulsion	3 mg/mL	67
	TWEEN 20, EtOH, water, vitamin E	Microemulsion	14.57 mg/mL	68
	Cremophor RH40, Transcutol P aqueous solution, Capryol 90	Microemulsion	32.5 mg/mL	69
	EtOH, triacetin, water	SFME (15% water)	77 mg/mL	13
	TWEEN 60, PBS, sodium alginate, MCT	Hydrogel nanoemulsion	67 µg/mL	70
	TWEEN 80, PBS, κ-carrageenan and alginate, corn oil	Hydrogel nanoemulsion	0.18 mg/mL	71
Gels	Whey protein isolate	Microgel	17.51 ± 0.46 µg/mg protein	72
	Chitin	Nanogel	0.475 mg/mL	73
	Fenugreek galactomannan	Hydrogel, commercial product (CurQfen)	0.316 mg/mg powder	74

Table 3.1 continued.

Carrier/Solubilization system	Reagent(s)	Comments	c(Cur)	Source
Phosphatidylcholine-based carrier systems	Sunflower lecithin, PBS	Nanoliposomes	0.6 mg/mL	75
	Soy lecithin, cholesterol, TWEEN 80, PBS	Nanoliposomes	0.24 mg/mL	76
	L- α -Phosphatidylcholine, cholesterol, PBS	Liposomes	0.89 mg/mL	77
	Soybean phospholipids, cholesterol, TWEEN 80, PBS	Liposomes	1.6 mg/mL	78
	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine, N-(carboxymethoxy)polyethylenglycol-2000)-1,2 distearoyl-sn-glycero-3-phosphoethanolamine, cholesterol, water	PEG-coated nano liposomes	4.7 mg/mL	79
Lipid-based nanoparticles	Phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, water	Liposomes	0.3 mg/mL	80
	Soybean-based phospholipids	Phytosomes	12.9 mg/mg	81
	Poly(ϵ -caprolactone), grape seed oil, sorbitan monostearate, polysorbate 80, water	Lipid-core nanocapsule	0.50 $\pm$ 0.02 mg/mL	82
Lipid-based nanoparticles	Trimyristin, soy lecithin, sodium glycocholate, poloxamer 407, glycerol	Solid lipid nanoparticles	0.05 mg/mL	83
	Murumuru butter, Compritol ATO888, Span20, Tween80	Solid lipid nanoparticles	500 μ g/mL	84
	Compritol R 888 ATO, GMS, polyethylenglycol 600, TWEEN 80, phospholipon 90G, water	Solid lipid nanoparticles	15 mg/mL	85
	Compritol ATO 888, sodium caseinate	Solid lipid nanoparticles	~19 μ g/mL	86
	MCT oil, TWEEN 80, glycerol tristearate, Span 80, water	Nanostructured lipid carrier	0.2 mg/mL	87

Table 3.1 continued.

Carrier/Solubilization system	Reagent(s)	Comments	c(Cur)	Source
Protein-based nanoparticles	Soy peptide	c(Soy peptide) = 1% (w/v)	0.1296 mg/mL	88
		c(SPI) = 3.5% (w/v)	1.085 mg/mL	89
		c(SPI) = 0.046% (w/v)	72 µg/mL	90
	Soy protein isolate (SPI)	c(SPI) = 1% (w/v)	8.9 µg/ml	91
		c(SPI) = 2% (w/v)	6.2 mg/mL	92
		c(SPI) = 0.5% (w/v)	0.2 mg/mL	93
		c(PPIp) = 6% (w/v)	60 µg/mL	94
	Pea protein isolate (PPIp)	c(PPIp) = 5% (w/v)	411.5 µg/mL	95
		c(PPIp) = 0.5% (w/v)	27.6 µg/mL	96
	Pea vicilin	c(Vicilin) = <1% (w/v)	0.47 mg/mL	97
	NaCas	c(NaCas) = 2% (w/v)	1.4 mg/mL	98
	Whey protein isolate (WPI)	c(WPI) = 0.9% (w/v)	0.85 mg/mL	99
		c(WPI) = 0.4% (w/v)	0.41 mg/mL	100
	Egg white protein isolate (EPI)	c(EPI) = 0.5% (w/v)	0.17 mg/mL	101
	Egg ovalbumin (Ova)	c(Ova) = 10^{-4} mol/L	32.73 µg/mL	102
	Bovine serum albumin (BSA)	c(BSA) = 0.5% (w/v)	29 µg/mL	103

3.2.2.2. Plant-Protein-Based Nanocomplexation of Curcumin

Soy is the most thoroughly researched plant-based protein source, and its effectiveness in solubilizing bioactive molecules is frequently documented with various pre-treatments^{44,104}. In particular, the ability to form complexes and nanoscale particles has attracted significant attention in recent research. To compare the performance of protein carriers regardless of concentration and processing method, the loading amount (LA) is often reported. This value is calculated as the ratio of solubilized Cur mass to the solubilized carrier mass⁹⁸.

Zhang *et al.* fabricated Cur-loaded soy peptide nanoparticles with a particle size of about 106 nm through sonication. They achieved a Cur solubility in water of 0.1296 mg/mL at a protein concentration of 1% (w/v), corresponding to a LA of 12.96 µg/mg soy peptide⁸⁸. Chen *et al.* prepared Cur nanocomplexes via a desolvation method. They dissolved Cur in EtOH and added it to a heated soy protein isolate (SPI) solution. The particle size was about 90 nm, and the Cur concentration in water was around 1.085 mg/mL with a SPI concentration of 3.5% (w/v). This corresponds to a LA of 31 µg/mg SPI⁸⁹. In another study, the authors examined the effect of sonication on the nanocomplexation of SPI with Cur. They reported an increased LA of 144.5 µg/mg SPI, corresponding to an aqueous Cur concentration of 72 µg/mL with a SPI concentration of 0.046% (w/v) and particle sizes of 110 nm⁹⁰. Tapal and Tiku reported the formation of SPI-Cur complexes by mixing SPI and Cur in water. At a SPI concentration of 1% (w/v), a Cur concentration of 8.9 µg/mL was achieved in water, corresponding to a LA of 0.89 µg/mg. The authors stated that redispersion of the lyophilized complex in larger quantities and, therefore, a higher concentration of Cur in water, is possible⁹¹. Wang *et al.* fabricated Cur-loaded SPI nanoparticles using a pH-driven method. An aqueous SPI dispersion was adjusted to pH 12, Cur was added, and then, the solution was readjusted to pH 7, resulting in particles smaller than 100 nm. The resulting Cur concentration in water was about 6.2 mg/mL with a SPI concentration of 2% (w/v), which corresponds to a LA of 309.11 µg/mg SPI⁹². Li *et al.* also produced nanocomposites of Cur and SPI using pH shifting. They compared the solubilization of Cur with untreated SPI and pH-shifted SPI, noting significantly improved performance when treating SPI with pH shifting, reaching a LA of about 33 µg/mg SPI. The highest Cur concentration was 0.2 mg/mL at a SPI concentration of 0.5% (w/v), with a particle size of about 80 nm⁹³. The pH shifting method was first introduced by Pan *et al.* to incorporate Cur into self-assembled casein nanoparticles. According to the authors, raising the pH to a highly alkaline level causes Cur to deprotonate and sodium caseinate to dissociate. By readjusting to a neutral pH, sodium caseinate reassembles into nanoparticles that enclose Cur. Applying this process, a Cur concentration of 1.4 mg/mL could be achieved with a carrier concentration of 2% (w/w) sodium caseinate, resulting in a LA of about 70 µg/mg sodium caseinate and in particles with a hydrodynamic diameter of around 100 nm⁹⁸. Since then, the pH-driven method has become a standard technique for

modifying (plant) protein structures and for binding and solubilizing lipophilic compounds. Compared to desolvation, pH shifting is solvent-free, more cost-effective, energy-efficient, and scalable. However, the components to be encapsulated must remain stable in the short term in alkaline media^{46,47,105}.

Soy protein is not the only one capable of solubilizing and stabilizing Cur. Zein protein^{106–110}, pea protein^{66,94–96}, rice protein¹¹¹, sunflower protein¹¹², and others have also shown promising results. Soy protein is appealing for human nutrition and related uses due to its well-balanced amino acid profile and good functional properties. However, its allergenic potential and frequent genetic modification are problematic. In comparison, pea protein is a hypoallergenic and clean-label alternative, and it is widely accessible^{113–116}.

Vijayan *et al.* examined the effects of pH, temperature, and pea protein concentration on the solubilization of Cur with untreated PPIp (simple complexation). The highest Cur solubility was achieved at 28 °C and neutral pH, with a concentration of around 12 µg/mL. Maintaining temperature and pH and increasing the protein concentration from 1% (w/v) to 6% (w/v), enhanced the Cur solubility to about 60 µg/mL. The LA increased from 1.2 µg/mg PPIp to 6.0 µg/mg PPIp, respectively⁹⁴. Ren *et al.* prepared nanoparticles of PPIp using a pH-driven method. At a PPIp concentration of 5% (w/v), they obtained a Cur concentration of 411.5 µg/mL, corresponding to a LA of about 33 µg/mg. The particle sizes were broadly distributed, with an average particle size of 250 nm⁹⁵. Guo *et al.* also fabricated nanocomplexes of PPIp Cur using a pH shifting method. They achieved a Cur concentration of 27.6 µg/mL at a PPIp concentration of 0.5% (w/v). The complexes had a particle size of 135 nm and a LA of 6 µg/mg PPIp. They further optimized the nanoparticles through complexation with surfactants⁹⁶. Liu *et al.* formed Cur-loaded pea vicilin nanoparticles with a particle size of 32 nm via a pH-driven method. At a carrier concentration of less than 1% (w/v), they obtained a Cur concentration of 0.47 mg/mL, corresponding to a LA of 82 µg/mg pea vicilin⁹⁷.

Various approaches have been used to understand how plant proteins stabilize and solubilize Cur. Molecular docking and simulation studies are primarily conducted with individual native protein fractions. Li *et al.* investigated the binding between Cur and SPI, using the main soy protein components β-conglycinin and glycinin to identify the optimal binding conformation of Cur at different pH levels⁹³. Ren *et al.* performed molecular dynamics simulations and molecular docking with the main fractions of pea protein, legumin, vicilin, and convicilin, to explore their interactions with Cur⁹⁵. Wang *et al.* predicted the native structure of 22 kDa alpha-zein 16 to examine its interactions with Cur through molecular dynamics and molecular docking analysis¹⁰⁸. Okagu and Udenigwe extracted albumin, globulin, and glutelin from pea and studied the binding of Cur to the individual pea protein fractions. They reported that glutelin showed the best performance due to its increased surface hydrophobicity and the resulting strong binding to Cur¹¹⁷. These fundamental studies have shown that

curcumin interacts with hydrophobic sites on proteins via non-covalent interactions, mainly hydrogen bonds and hydrophobic forces, leading to conformational modifications in the protein structure and stabilization of Cur^{93,95,108}. These studies assume a native protein structure, in which its functionality determines access to binding sites and how the bound compound interacts with the surrounding environment¹¹⁸. However, these structural and functional attributes are often significantly compromised in commercial protein powders. Due to the harsh conditions typically employed during extraction and drying, such powders consist of heterogeneous protein mixtures that may include residual impurities, such as fibers and minerals, and are largely denatured, leading to a substantial loss of functionality (discussed in detail in section 2.2.5.1)^{116,119,120}. This situation alters the considerations necessary for mechanism determination when using commercial products. Some studies have applied the knowledge gained from fundamental research to the more common case of commercial protein isolates. Zhan *et al.* presented a schematic mechanism for the pH-driven formation of Cur-loaded whey protein isolate (WPI) and zein composite nanoparticles, supported by infrared spectroscopy and X-ray diffraction analysis. They summarized that Cur is loaded into WPI and composite nanoparticles via the unfolding and refolding of WPI upon pH shifting. Both Cur and zein are deprotonated and dissolve in an alkaline environment, then interact through hydrophobic interactions. When the pH is adjusted to 7, WPI refolds and encapsulates Cur or Cur-zein particles⁹⁹. Teng *et al.* described a schematic mechanism for encapsulating Cur in SPI nanoparticles via desolvation. They assumed that SPI exists in a native state with a few larger aggregates and that hydrophobic chains are exposed when EtOH is added. When introducing the ethanolic Cur solution, Cur binds both inside and outside the resulting nanoparticles to hydrophobic binding sites. The authors support their proposed mechanism with results from X-ray diffraction analysis and scanning electron microscopy¹²¹. Wang *et al.* studied the pH-driven binding of Cur to soy protein using fluorescence quenching and isothermal titration calorimetry. They identified hydrophobic interactions as the main force between Cur and soy protein and concluded that pH shifting provided better access for Cur to the binding sites on the protein⁹².

In two comprehensive reviews, Chuan-He Tang summarized that the binding of Cur to proteins is well-documented for native, thoroughly characterized proteins, such as caseins and serum albumins. However, the binding of commercially available, mostly denatured plant proteins is less studied and understood^{44,46}. According to current research, the existing literature has not yet documented the fabrication of kinetically stable, supersaturated nanoparticles through a straightforward method of pH shifting and thermal processing. To better assess the potential of Cur dissolved by plant proteins, a more detailed investigation into the solubilization mechanism of Cur with a commercial pea protein isolate is presented in the following, along with strategies to stabilize supersaturation. The factors discussed above are summarized in Figure 3.3.

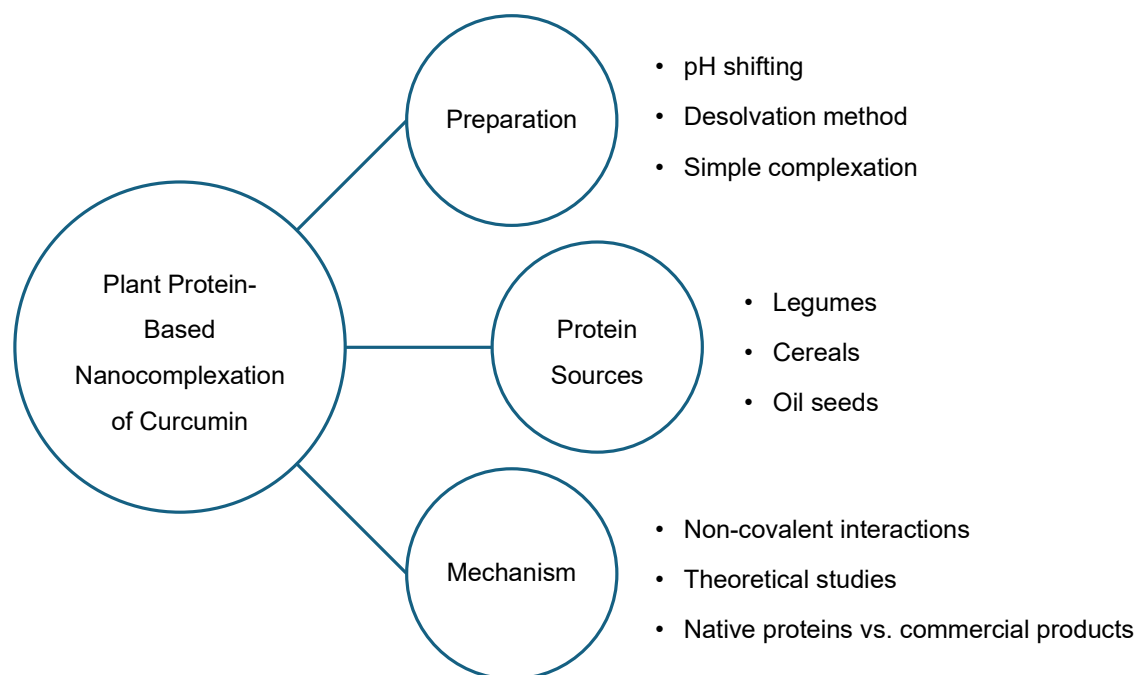


Figure 3.3: Overview of different preparation methods for nanocomplexation of Cur with plant proteins, protein sources, and approaches to understanding the binding mechanism of Cur to protein carriers. The listed protein sources are the most used ones.

3.3. Experimental Section

3.3.1. Materials

Curcumin (Cur, synthetic, >97.0%), bisdemethoxycurcumin (BDMC, >98.0%), ferulic acid (>98 %), vanillic acid (> 98%), and *para*-hydroxybenzaldehyde (>98.0 %) (GC) were purchased from TCI Chemicals (Eschborn, Germany). Vanillin (>97 %), sodium azide (>99.5 %), and bile salts (suitable for microbiology) were obtained from Sigma-Aldrich (Darmstadt, Germany). Pea protein isolate NUTRALYS® F85F (PPIp, ≥93%, batch WE802) was provided by Roquette (Lestrem, France). Sodium hydroxide solution (NaOH, 1 N), ethanol (EtOH, ≥99.8%, p.a.), sodium chloride (NaCl, ≥99.5%), potassium chloride (KCl, ≥99%), potassium dihydrogen phosphate (KH₂PO₄, ≥99%), sodium hydrogencarbonate (NaHCO₃, ≥99%), magnesium chloride hexahydrate (MgCl₂(H₂O)₆, ≥99%), and ammonium carbonate (≥30.5% NH₃) were purchased from Carl Roth (Karlsruhe, Germany). Hydrochloric acid (HCl, 1 N) and calcium chloride (CaCl₂, ≥93.0%) were obtained from Merck KGaA (Darmstadt, Germany). MilliQ water (18 MΩ·cm) was generated using an in-house MilliQ purification system.

3.3.2. Methods

3.3.2.1. Preparation of Pea Protein Isolate Nanoparticles (PPI)

Pea protein isolate nanoparticles (PPI) were prepared using a pH shifting and heating protocol inspired by the methods of Han *et al.*¹²² and Pan *et al.*⁹⁸: PPIp was dispersed in MilliQ water at 1% (w/v) (10 mg/mL) and stirred for 30 minutes at 22 °C. The pH was initially adjusted to 12.00 ± 0.05 using NaOH (1N), followed by stirring for 30 min. Subsequently, the solution was placed in a water bath at 85 °C and maintained under continuous stirring for an additional 30 min. After cooling to 22 °C, the pH was adjusted back to 7.00 ± 0.05 with HCl (1N). The freshly prepared sample was designated as t0 and was either analyzed immediately or stirred further for 15 h at 22 °C. The next day, the sample was centrifuged (20379 × g, 15 min) and labelled as t24. All experiments were performed in triplicate.

3.3.2.2. Preparation of Pea Protein Isolate Nanoparticles Loaded with Curcumin (PPICur)

Pea protein nanoparticles loaded with Cur (PPICur) were prepared following the same protocol described above, including loading with Cur. The PPI concentrations used ranged from 0.2% to 3.0% (w/v). After heating and cooling, the pH was reset to 12.00 ± 0.05. The solutions were loaded with varying amounts of Cur, resulting in $\beta_{\text{load}}(\text{Cur}) = 0.5\text{--}16$ mg/mL, and stirred for 20 min at 22 °C. The pH

was then readjusted to 7.00 ± 0.05 with HCl (1 N) and stirred for five minutes. Undissolved Cur was removed by centrifugation ($20379 \times g$, 15 min). The supernatant (PPICur) was filtered through a cellulose acetate (CA) filter with a $0.45 \mu\text{m}$ pore size. The freshly prepared sample was designated as t_0 and was either analyzed immediately or stirred further for 15 hours at 22°C . The next day, the sample was centrifuged, and the supernatant (PPICur) was filtered again (CA $0.45 \mu\text{m}$) and designated as t_{24} . All experiments were performed in triplicate.

In one experiment, the heating step was skipped, and the sample was stirred for the appropriate duration at room temperature (RT).

3.3.2.3. Determination of the Solubility and Stability of Curcumin in PPICur

High-performance liquid chromatography (HPLC) and UV-Vis spectroscopy were performed to evaluate the solubility and chemical stability of Cur. A volume of 2 mL of PPICur was extracted with 25 mL of EtOH (20 mL in a first extraction cycle and 5 mL in a second extraction cycle). PPI precipitated and was separated by centrifugation. Both extraction volumes were combined and filtered using a PTFE filter with a pore size of $0.2 \mu\text{m}$. The Cur concentration was analyzed by UV-Vis spectroscopy at 427 nm using a PerkinElmer Lambda18 spectrophotometer (Überlingen, Germany) with quartz cuvettes of 1.000 cm path length. A calibration curve for Cur in EtOH with the equation $y = 56499x$ ($R^2 = 0.99994$) was prepared (see Figure SI 3 1). Samples were prepared in triplicate, and mean values and standard deviations were calculated.

The chemical stability of Cur was monitored using HPLC. The EtOH extracts and the precipitate (resolubilized in EtOH), were analyzed using a HPLC system from Waters (Eschborn, Germany) equipped with an Altmann Analytik Nucleosil C18 $5 \mu\text{m}$ HPLC column (Munich, Germany), a Gynkotek STH 585 column oven, a Waters 515 HPLC pump, a Waters pump control module II, a Waters 717plus autosampler, and a Waters 2996 photodiode array detector. A sample volume of $10 \mu\text{L}$ was injected and eluted at a 0.4 mL/min flow rate and at 25°C . All samples were measured in duplicates. Two different mobile phase compositions were used to separate either Cur or a series of known degradation products (vanillin, vanillic acid, ferulic acid, and *p*-hydroxybenzaldehyde) isocratically. Cur was eluted with a 60/40 mixture of 60% methanol and acetonitrile (1:1 v/v) and 40% trifluoroacetic acid at a concentration of 0.1% (v/v) in water. The degradation products were eluted with the same solutions in a 30/70 ratio (30% methanol and acetonitrile, 70% aqueous trifluoroacetic acid solution), inspired by Sinha *et al.*¹²³. The raw data were processed with the Empower 3 software. Chromatograms of Cur and vanillin are provided in Figure SI 3 2. In addition to HPLC, the precipitate was analyzed using catalytic tube combustion with oxygen supply employing an elemental analyzer vario MICRO Cube from Elementar Analysensysteme GmbH (Langenselbold, Germany). The analysis was conducted at the central analytical department of the University of Regensburg.

3.3.2.4. Determination of Protein and PPIp Solubility

The solubility of PPIp was evaluated as a mass concentration using equation (3.1).

$$\beta(PPIp) \left[\frac{mg}{mL} \right] = \frac{m(PPIp) - m(residue)}{V(H_2O)} \quad (3.1)$$

with $m(PPIp)$ being the used mass of PPIp given in mg, $m(residue)$ being the mass of the residue given in mg, and $V(H_2O)$ being the volume of water in mL.

The pea protein solubility was determined as a relative solubility by calculating the NSI (see Chapter 2, equation 2.3). NSI was then converted into a protein mass concentration (see Chapter 2 equation 2.4). Assuming NSI=1, i.e., the complete solubilization of the protein, from a dispersion of 10 mg of PPIp in 10 mL of water, and considering the nitrogen content of PPIp, the maximum protein mass concentration is 7.76 ± 0.0094 mg/mL.

Solubility of PPIp and protein content were monitored during the preparation and after 24 hours. After each step in the protocol, samples were centrifuged, lyophilized, and analyzed using catalytic tube combustion with oxygen supply. All samples were prepared in triplicate and analyzed up to three times. The mean values and standard deviations thereof were calculated.

3.3.2.5. Characterization of PPI and PPICur

Binding efficiency (BE) and loading amount (LA): The solubilization performance of PPI was evaluated in terms of binding efficiency (BE) and loading amount (LA). BE and LA were determined using the following equations (3.2) and (3.3):

$$BE [\%] = \frac{\beta(Cur)_{sol}}{\beta(Cur)_{input}} \cdot 100 \quad (3.2)$$

$$LA [\%] = \frac{\beta(Cur)_{sol}}{\beta(PPI)} \cdot 100 \quad (3.3)$$

with $\beta(Cur)_{sol}$ being the mass concentration of Cur solubilized by PPI, $\beta(Cur)_{input}$ indicating the mass concentration of Cur during loading, and $\beta(PPI)$ indicating the mass concentration of PPI.

Preparative size exclusion chromatography (SEC): SEC was performed at room temperature using a HiLoadTM 16/600 Superdex 200 pg column in TRIS buffer (20 mM TRIS pH 7.5, DTT, 150 mM NaCl). A sample volume of 2 mL was loaded onto the column with a flow rate of 0.5 mL/min. For sample analysis, the flow rate was increased to 1 mL/min, while the elution from the column was monitored with UV-Vis detection at $\lambda = 280$ nm.

Mass spectrometry (MS): samples were subjected to trypsin digestion, separated via C18 reversed-phase chromatography, and measured online via ESI-QTOF. The Uniprot database of *Pisum sativum* was used for data evaluation. Further information is provided in SI Chapter 3.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE experiments were performed as described in Chapter 2, section 2.3.3.1.

Zeta (ζ) potential: PPICur and PPI were diluted to a concentration of 0.5 mg/mL with an ionic strength of 1.16×10^{-3} M using MilliQ water. PPIp was diluted to 0.15 mg/mL, and an equivalent amount of NaCl was added to match the ionic strength of PPICur and PPI. ζ was measured using a ZetaSizer Nano ZS from Malvern Instruments GmbH (Kassel, Germany). All samples were prepared in triplicate and analyzed three times, with mean values and standard deviations calculated.

Nanoparticle Tracking Analysis (NTA): particle sizes were determined using a NanoSight NS300 device from Malvern Panalytical GmbH, Kassel, Germany. Samples were diluted to 20-100 particles per frame using MilliQ water and manually injected into the measurement flow cell. Particle tracks were recorded for 60 seconds per measurement, and each sample was measured three times ($N=3$). Particle hydrodynamic diameters are given in mean (average particle diameter) and mode (particle diameter with the highest particle concentration). The detection threshold was set to 4. The polydispersity index (PDI) was calculated using equation (3.4) from Clayton *et al.*¹²⁴, considering the error propagation.

$$PDI = \left(\frac{\sigma}{d} \right)^2 \quad (3.4)$$

with σ indicating the standard deviation of the particle diameter distribution and d indicating the mean particle diameter.

3.3.2.6. UV-Vis Spectroscopy of PPICur

PPI was used as a blank for UV-Vis spectroscopy of PPICur using a Lambda18 spectrophotometer from PerkinElmer (Überlingen, Germany). Measurements were performed in disposable micro-UV-cuvettes from Brand (Wertheim, Germany) with a path length of 0.100 cm. Ethanolic solutions were measured in quartz cuvettes with a path length of 1.000 cm. To measure the kinetic profiles, UV-Vis spectra were recorded at specified time intervals with the solution medium as the blank. Before each measurement, samples were filtered (CA 0.45 μ m).

The simulated intestinal fluid (SIF) used in section 3.4.5 was prepared according to the international consensus reported by Minekus *et al.*¹²⁵, but without enzymes. The following aqueous salt solutions were mixed in a 200 mL volumetric flask (concentrations of the solutions are given in brackets): 3.4 mL KCl solution (0.5 M), 0.4 mL KH_2PO_4 solution (0.5 M), 21.25 mL NaHCO_3 solution (1 M), 4.8 mL NaCl solution (2 M), and 0.55 mL $\text{MgCl}_2(\text{H}_2\text{O})_6$ (0.15 M). The remaining volume was filled with MilliQ water.

The pH of the solution was set to 7 using HCl (6 M). A volume of 120 mL of the salt solution was taken and mixed with 0.6 mL CaCl₂ solution (0.075 M) and 15 mL bile salts solution (0.1 M). A volume of 1.5 mL MilliQ water was added.

3.3.2.7. Fluorescence Spectroscopy of PPICur

The fluorescence of PPICur was measured using an Infinite M Nano+ from Tecan Trading (Männedorf, Switzerland). The samples were measured in a flat-bottom black Brand® 96-well microplate with a gain of 100 in top reading mode and bandwidths of 20 (emission) and 5 (excitation). Emission spectra were recorded between 430 nm and 800 nm with excitation wavelengths between 390 nm and 450 nm. Excitation spectra were recorded between 300 nm and 500 nm with emission wavelengths ranging from 500 nm to 550 nm. A stock solution of Cur in MeOH at 5 mM was prepared. All samples were diluted to match absolute concentrations. PPICur was diluted with PPI solution and Cur was diluted with MilliQ water.

3.3.2.8. X-Ray Diffraction (XRD)

Samples t0 and t24 were lyophilized for 48 hours and the resulting powders were ground in a mortar. Analysis was carried out using transmission geometry on a STOE STADI P diffractometer (Stoe & Cie) equipped with a Mythen 1K detector for data collection and CuK α 1-radiation ($\lambda = 1.540598 \text{ \AA}$). Raw data were processed using the WinXPOW software package from Stoe & Cie (Vol. Version 3.0.2.5)¹²⁶.

3.3.2.9. Circular Dichroism (CD)

CD spectra of PPIp, PPI, and PPICur were recorded on a J-710 spectropolarimeter from Jasco (Pfungstadt, Germany). Samples were diluted to a concentration of 0.14-0.15 mg/mL with MilliQ water. The CD spectra were measured over a wavelength range of 185-260 nm using quartz cuvettes with a path length of 0.100 cm. The bandwidth and step size were 1.0 nm and 0.5 nm, respectively, and the scan speed was 100 nm/min. Ten spectra per sample were averaged and the baseline spectra of the blank were subtracted before data processing. The secondary structure proportions were evaluated using a statistical amino acid molar mass of 115 Da and DichroWeb and the CDSSTR algorithm with the reference set 6¹²⁷.

3.4. Results and Discussion

3.4.1. Solubility and Stability of Curcumin

PPICur t0 was analyzed using UV-Vis spectroscopy at specific intervals within eight hours after preparation, then again at 24 hours (designated as PPICur t24) and 48 hours. Two samples were compared: one was stirred during the observation period (filled symbols in Figure 3.4), and the other was not (unfilled symbols in Figure 3.4). The concentration of PPIp (β (PPIp)) was 1% (w/v) and the concentration of Cur during loading was 8 mg/mL ($\beta_{\text{load}}(\text{Cur})$). The courses of the absorbance are shown as normalized values (A/A_0) in Figure 3.4 A. Within eight hours, the absorbance of Cur decreases by about 18% in the stirred solution. After 24 hours, the absorbance decreases by 96%, accompanied by the appearance of an orange precipitate. In the case of the unstirred sample, the absorbance decreases by 18% within 24 hours and by 28% within 48 hours. The visual appearances of PPICur t0 and t24 are shown against white and black backgrounds in Figure 3.4 B. While PPICur t0 is translucent, PPICur t24 (stirred) is clear. The state of PPICur t0 could not be changed through centrifugation and filtration.

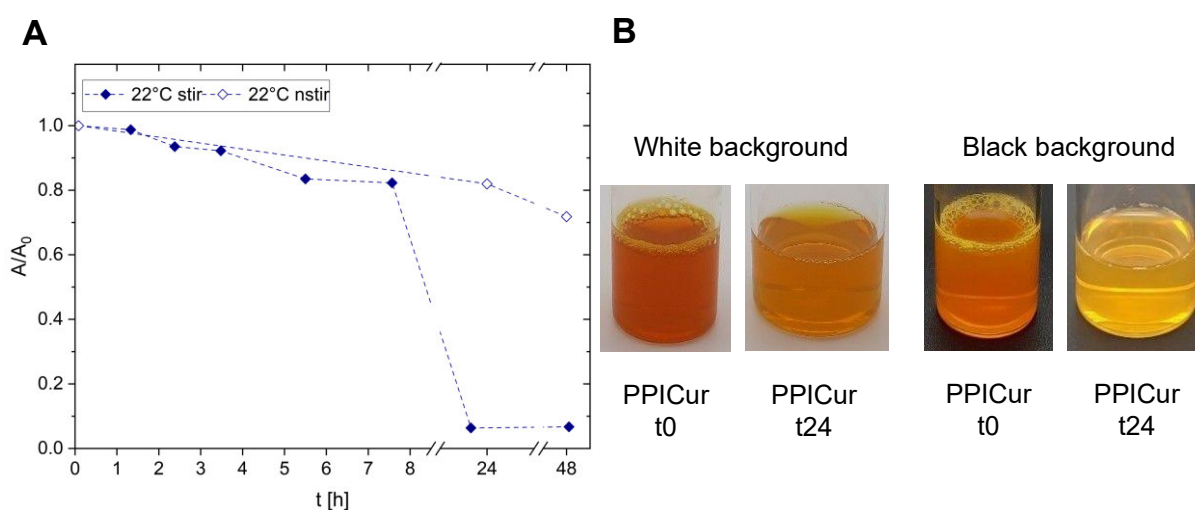


Figure 3.4: Time course of the normalized absorbance of PPICur t0 stirred and unstirred at 22 °C at β (PPIp) of 1% (w/v) and $\beta_{\text{load}}(\text{Cur})$ of 8 mg/mL measured through UV-Vis spectroscopy (A). Photos of PPICur t0 and PPICur t24 (stirred) against white and black backgrounds (B).

HPLC analysis of the ethanolic extracts of PPICur was performed to examine their composition in relation to Cur degradation (UV-Vis spectra of the degradation products vanillin, vanillic acid, ferulic acid, and *para*-hydroxybenzaldehyde, see Figure SI 3 3). Cur was identified as the primary compound in both samples t0 and t24. Traces of vanillin were detected in both the pure Cur powder during calibration (see Figure SI 3 2) and in the PPICur extracts (see Figure SI 3 4). HPLC analysis of the orange precipitate, which was fully redispersed in EtOH, revealed Cur and traces of vanillin as the only components (see Figure SI 3 5). The presence of undetected degradation products was eliminated by

mass balancing the used, solubilized, and precipitated Cur. Consequently, no degradation of Cur was observed within 24 hours, and the decrease in absorbance was attributed to its precipitation.

The effect of parameters $\beta(\text{PPIp})$ and $\beta_{\text{load}}(\text{Cur})$ on the Cur concentration in PPICur was examined using UV-Vis spectroscopy of ethanolic extracts of PPICur at t_0 and t_{24} and the calibration curve of Cur in EtOH (see Figure SI 3 1). The optimal loading time was found to be 20 min (see Figure SI 3 6). Cur concentrations obtained with various $\beta(\text{PPIp})$ and $\beta_{\text{load}}(\text{Cur})$ values are summarized in Figure 3.5.

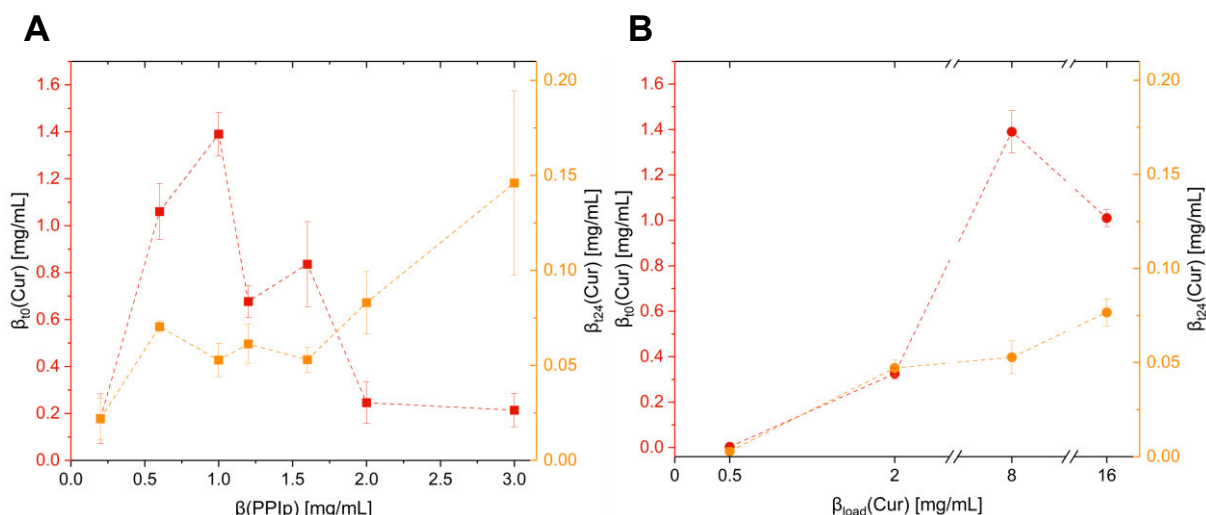


Figure 3.5: $\beta_{t0}(\text{Cur})$ and $\beta_{t24}(\text{Cur})$ of PPICur at varying $\beta(\text{PPIp})$ (A) and $\beta_{\text{load}}(\text{Cur})$ (B). In A, $\beta_{\text{load}}(\text{Cur})$ is kept constant at 8 mg/mL. In B, $\beta(\text{PPIp})$ is kept constant at 1% (w/v).

Varying $\beta(\text{PPIp})$ from 0.2 to 3.0 mg/mL at a constant $\beta_{\text{load}}(\text{Cur})$ of 8 mg/mL (A) initially increases the concentration of Cur in PPICur at t_0 , then decreases it. The maximum $\beta_{t0}(\text{Cur})$ of 1.39 ± 0.0930 mg/mL is observed at $\beta(\text{PPIp}) = 1\%$ (w/v). The Cur concentration after 24 hours increases with higher PPI concentrations, reaching a maximum of $\beta_{t24}(\text{Cur}) = 0.146 \pm 0.0485$ mg/mL at $\beta(\text{PPIp}) = 3\%$ (w/v). Varying $\beta_{\text{load}}(\text{Cur})$ from 0.50 to 16 mg/mL (B) at a constant $\beta(\text{PPIp})$ of 1% (w/v) initially increases the Cur concentration in PPICur at t_0 , then decreases it. After 24 hours, the Cur concentration increases with higher $\beta_{\text{load}}(\text{Cur})$, peaking at $\beta_{t24}(\text{Cur}) = 0.0766 \pm 0.00718$ mg/mL at $\beta_{\text{load}}(\text{Cur}) = 16$ mg/mL. Vijayan *et al.* used a commercial PPIp from Roquette India to solubilize Cur and examined the effect of different $\beta(\text{PPIp})$ levels on the concentrations of solubilized Cur. They observed an increase in Cur concentrations with rising $\beta(\text{PPIp})$, reaching a maximum of about 60 $\mu\text{g/mL}$ at a $\beta(\text{PPIp})$ of 6% (w/v). The complexation was achieved simply by mixing PPIp and Cur⁹⁴.

Larger $\beta(\text{PPIp})$ values were not tested here because the samples exhibited increased viscosity at $\beta(\text{PPIp}) = 3\%$ (w/v). This may also explain the lower $\beta_{t0}(\text{Cur})$ and higher $\beta_{t24}(\text{Cur})$ at this PPIp concentration. The increased viscosity could hinder Cur binding during loading and, to some extent, prevent the bound Cur from precipitating. Increasing $\beta(\text{Cur})$ in protein complexes with increasing $\beta_{\text{load}}(\text{Cur})$ is widely documented^{89,92,95,98}. However, it can be inferred from Figure 3.5 B that the

apparent $\beta(\text{Cur})$ corresponds to a supersaturation at t_0 . Since the kinetic stability and thus $\beta_{t24}(\text{Cur})$ is not documented, comparing to absolute values is difficult. The loading concentrations range from 0.5 to 8 mg/mL and are within the reported literature range. Especially, for high $\beta_{\text{load}}(\text{Cur})$, precipitation should have been observed, as supersaturated systems precipitate faster the higher the supersaturation¹²⁸. At $\beta_{\text{load}}(\text{Cur}) = 16 \text{ mg/mL}$, a decrease in $\beta_{t0}(\text{Cur})$ is observed. This can probably be explained by a reduced colloidal stability of the carrier in the presence of such a high amount of Cur^{46,129}.

The impact of the heating step during the preparation of PPICur on the final Cur concentration was also evaluated. $\beta(\text{PPIp})$ and $\beta_{\text{load}}(\text{Cur})$ were maintained at 1% (w/v) and 8 mg/mL, respectively. Cur concentrations after preparation and after 24 hours, as well as the BE and LA, are compared in Table 3.2. Heating to 85 °C before loading with Cur increases $\beta_{t0}(\text{Cur})$ and $\beta_{t24}(\text{Cur})$, and consequently, BE and LA. Heating is reported to enhance the surface hydrophobicity of proteins, leading to improved interaction with Cur^{117,130}.

Table 3.2: Cur concentration, BE, and LA after preparation and after 24 hours of PPICur, when PPI was prepared by pH shifting and heating to 85 °C, and pH shifting and stirring at RT.

	pH shifting + 85 °C		pH shifting + RT	
	PPICur t_0	PPICur t_{24}	PPICur t_0	PPICur t_{24}
$\beta(\text{Cur})$ [mg/m]	1.39 ± 0.0930	0.0528 ± 0.00878	0.663 ± 0.0256	0.0297 ± 0.00286
BE [%]	17.41 ± 1.28	0.695 ± 0.109	8.42 ± 0.327	0.360 ± 0.0332
LA [%]	14.27 ± 0.0276	0.615 ± 0.00387	6.68 ± 0.0541	0.305 ± 0.00354

LA values in the literature are presented in both percentage and mass-to-mass units. A LA of 14.27% equals 0.1427 mg/mL. The LAs obtained here (both at t_0 and t_{24}) fall within the range documented in the literature (see discussion in 0). A direct comparison of the performances of the protein carriers reported in the literature is difficult because the kinetic stability of the prepared complexes is not discussed, and the raw materials vary. The BE is also often specified together with the LA, but it is less meaningful because it largely depends on $\beta_{\text{load}}(\text{Cur})$ (see Figure 3.5).

3.4.2. Characterization of PPICur

In all subsequent experiments, PPICur was always prepared with $\beta(\text{PPIp})$ at 1% (w/v) and $\beta_{\text{load}}(\text{Cur})$ at 8 mg/mL. PPICur t_{24} always refers to the stirred version and is therefore no longer explicitly added.

pH shifting and heating during the preparation of PPICur certainly cause some degree of aggregation in the protein¹³¹. The overall size distribution in PPIp, influenced by the preparation protocol and Cur loading, was analyzed using SDS-PAGE and size-exclusion chromatography (SEC). The results are shown in Figure 3.6. SDS-PAGE of PPIp shows distinct bands across the entire range of the molecular weight standard. A low-intensity peak at the edge of the dead volume (around 42 mL) and a broader, higher-

intensity peak between 90 and 125 mL are observed in the SEC analysis. The SEC analysis of PPICur t0 and t24 reveals in both cases a high-intensity peak near the dead volume (1), two smaller, less-defined peaks at 65-70 mL and 85 mL (2, 3), and a peak at 110 mL (4). Based on the optical appearance of the collected fractions of PPICur t0 and t24, only the protein fractions producing the first two peaks are colored. These are summarized in fractions I (colored) and II (non-colored). Within 24 hours, peak 1 decreases, while peak 2 increases. Peaks 3 and 4 remain nearly unchanged. SDS-PAGEs of PPICur t0 and t24 appear identical, showing an intense band above the molecular weight marker for fraction I and two bands around 25 kDa for fraction II. Peaks 1 and 2 are too large to assign a molecular weight, as they do not migrate into the separation gel in the SDS-PAGE and elute with or near the dead volume of the SEC. Adjusting SDS-PAGE analysis by using a larger gel pore size and treating samples with a high-salt buffer did not alter the results (data not shown). According to the SEC column's separation limit, these fractions are larger than 600 kDa. The fraction larger than 600 kDa is already present in PPIp and becomes more pronounced during PPICur preparation. The SEC profile is similar for PPI without Cur loading (see Figure SI 3 7). Since only fraction I is colored, it is concluded that exclusively the large protein aggregates solubilize Cur, while the smaller ones do not participate in the binding of Cur.

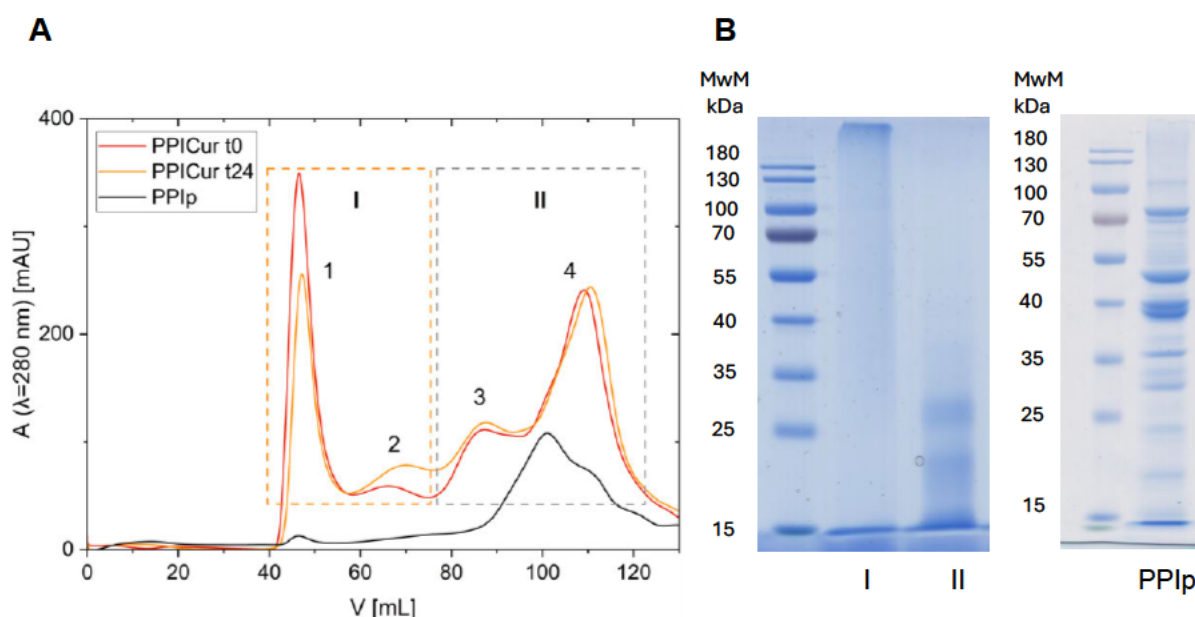


Figure 3.6: Chromatograms of PPICur t0, and t24, and PPIp obtained from SEC (A). Peaks 1 and 2 of PPICur t0 and t24 are summarized in fraction I (colored) and peaks 3 and 4 are summarized in fraction II (not colored). Electrophoretic patterns of fractions I and II and PPIp (B). PPIp was run before SEC analysis, and fractions I and II were run afterwards.

Mass spectrometry (MS) of fractions I and II of PPI and PPICur showed that they contain protein mixtures with no significant compositional differences (see MS data in Table SI 3 1). Due to their size, it can be assumed that all peaks represent aggregates without a specific affinity of Cur for particular proteins in the mixture. The only decisive factor is the aggregate size. Considering these results, the LA can also be expressed as a fractionated LA, based solely on the PPI proportion involved in Cur binding,

which was calculated from the areas under the SEC curve (see Table 3.3, SEC areas see Figure SI 3 8). The values of LA_{frac} indicate that at t_0 , about 37% of the PPI fraction is involved in Cur binding, and at t_{24} , only about 2% of that fraction is covered with Cur.

Table 3.3: Loading amounts (LA) of PPICur t_0 and t_{24} considering the total mass of PPI (LA_{tot}) and the fraction included in the binding of Cur according to SEC (LA_{frac}).

	PPICur t_0	PPICur t_{24}
LA_{tot} [%]	14.27 ± 0.0276	0.615 ± 0.00387
LA_{frac} [%]	37.2 ± 0.0720	1.76 ± 0.0111

When analyzing the aggregates and the binding of Cur to those, several factors concerning the preparation must be considered: heat treatment causes aggregation and gelling of proteins^{132–134}. The heat denaturation temperature (T_d) and the gelation concentration (β_g) of proteins are two key parameters that influence these phenomena. T_d describes the unfolding of native structures above a specific temperature¹³⁵. Shand *et al.* reported a T_d of 85 °C for native pea proteins¹³⁵. Since most commercial PPIp is denatured, T_d relates more to the breaking and unfolding of aggregates^{44,45}. The temperature that marks the beginning of gel formation is described as the gelling temperature (T_g)¹³⁶. T_g of commercial PPIps, at a concentration above β_g , has been reported between 82–93 °C^{134,135}. Regarding protein concentration, aggregates are formed below β_g , and a gel network develops above this concentration. β_g depends on pH, ionic strength, and protein functionality and composition. It is therefore also influenced by the extraction method and the source of the raw material^{132,133}. Gel formation of commercial PPIps was reported for β_g between 14.5–19.6% (w/w), at pH 5.65–7.10 and around 0.3 M NaCl, and heating to 82–93 °C^{134,135}. For PPIp dispersions at pH 5.65 with a concentration of 14.5% (w/v), T_g increased from 60 °C to 94 °C when the ionic strength was raised to 1.5 M NaCl. At 2 M NaCl, gel formation was suppressed. Keeping the concentration constant and at an ionic strength of 0.3 M, T_g rose from 56 °C to 89 °C as the pH increased from 3 to 6, decreased from 87 to 59 °C as the pH increased further to pH 10, and was 85 °C at pH 11¹³⁶.

In the presented case, the PPIp concentration is 1% (w/v) (10 mg/mL), the heating temperature is 85 °C, and the ionic strength is 2.3×10^{-2} M. Considering these factors, with particular regard to the used concentration, it is concluded that soluble heat-denatured protein aggregates are formed rather than a protein gel. This is also supported by the fact that PPICur t_0 and t_{24} were highly dilutable, excluding the presence of a stabilizing gel network (data not shown). The formation of compact, soluble heat-induced aggregates has also been suggested in the literature by Messian *et al.*, who extracted globulins from pea flour and denatured them by heating to 92 °C. They also conducted SDS-PAGE and SEC and observed similar results¹³⁷. In another study of Peng *et al.* SEC analysis revealed an increase in intensity in the first peak when comparing unheated and heated pea protein isolates. They also attributed that result to the formation of heat-induced, soluble aggregates¹³⁸.

To further characterize the aggregates, zeta (ζ) potential measurements and nanoparticle tracking analysis (NTA) were performed to determine the surface charges and particle sizes of PPIp, PPI, and PPICur t0 and t24 (see Figure 3.7 **A**, **B**, and **C**). The solubilities of PPIp and PPI were monitored throughout the entire preparation (D).

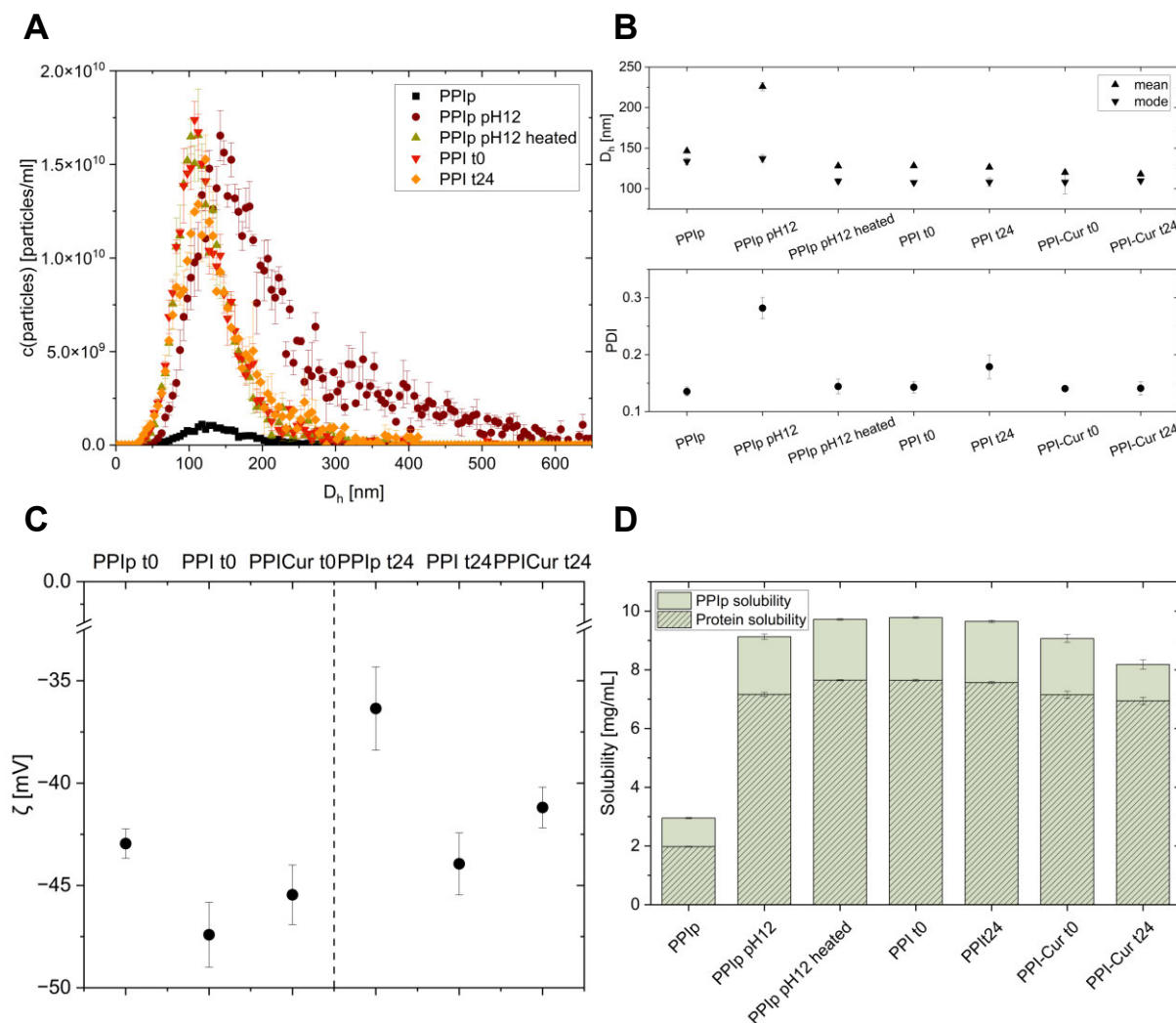


Figure 3.7: Particle size distribution (A), average particle sizes and PDI (B), zeta (ζ) potential (C), and PPIp and protein solubility (D) of the single steps of the preparation protocol and PPICur t0 and t24.

The solubility of PPIp in water after stirring for 30 min is about 3 mg/mL. The average (mean) particle size is 147 nm with a PDI of 0.14. After adjusting the pH to 12, the solubility increases to 9.1 mg/mL, and the size distribution broadens with diverging values for mean and mode particle sizes, leading to a higher PDI. Heating to 85 °C increases solubility to 9.7 mg/mL and reduces the particle size to a mean of 128 nm and a comparably low PDI. Particle size and solubility remain unchanged after cooling and readjusting to pH 7. The solubility of PPIp, PPI, and the protein content exhibits consistent behavior. Nearly 99% of PPIp is solubilized using pH shifting and heating treatment. Within 24 hours, about 1% of the protein precipitates. In the case of loading with Cur, about 7% of the protein precipitates immediately after cooling. Within 24 hours, 10% of PPI, comprising 3% protein, precipitates alongside

Cur. This is the only case where PPI and protein solubility diverge. The preparation yields a final PPI concentration of 8.20 mg/mL, including 6.94 mg/mL of protein.

By setting a highly alkaline pH level well above the isoelectric point of the storage proteins, ionizable side chains become deprotonated, resulting in repulsive electrostatic interactions. In native proteins, globular structures unfold, while in protein aggregates, these structures are disrupted. In both scenarios, the structures become more hydrated, and hydrophobic binding sites are exposed^{44,131}. The high charge can cause aggregates not only to disrupt but also to elongate, similar to a polyelectrolyte in water. This explains the difference between the mean particle size (226 nm) and the mode (137 nm), as well as a higher PDI. Wei Wang discussed protein aggregation and how different stimuli affect protein structure. Tang *et al.* examined the thermal aggregation of soy protein, and Chen *et al.* studied its effect on the performance of soy protein to bind Cur. According to them, heating induces further unfolding and increases surface hydrophobicity because of the greater exposure of hydrophobic binding sites. Chemical cross-links are also probable^{89,130,139}. Following these explanations, the reduction in particle size can be explained by the hydrophobic effect: large, loose structures dehydrate and shrink into compact aggregates, minimizing thermodynamically unfavorable interactions between surrounding water and hydrophobic surfaces¹⁴⁰. Electrostatic repulsion is the predominant contribution, suppressing aggregation caused by hydrophobic interactions¹⁴¹. Readjusting to pH 7 results in a partial neutralization of the protein surface. The increased protein and PPIp precipitation when loaded with Cur could be due to the decreased colloidal stability of protein particles when exposed to a high amount of Cur^{46,129}.

The absolute ζ value increases from -43 mV (PPIp) to -47 mV (PPI) due to pH shifts and heating, and is not significantly affected by loading with Cur. According to the argumentation of Pan *et al.*, the increase in the absolute ζ value can be attributed to the disruption of the protein aggregate supramolecular structure and the subsequent formation of nanoaggregates⁹⁸. Cur loading does not affect the nanoparticles' surface charge. Within 24 hours, all absolute ζ values consistently decrease. $\zeta(\text{PPIp})$ significantly drops to -36 mV, and $\zeta(\text{PPI t24})$ and $\zeta(\text{PPICur t24})$ decrease to -44 mV and -41 mV, respectively, indicating that the precipitation of Cur does not account for the decrease in ζ . The other way around— whether changes in surface properties cause Cur precipitation — remains uncertain, based on current results. Since the particle size remains largely unchanged, aggregation is unlikely to be a significant factor. Preserving bacterial growth and, thus, potential degradation of organic material by adding sodium azide did not change the precipitation behavior of Cur (data not shown).

To assess whether dynamic protein folding could cause Cur precipitation, circular dichroism (CD) measurements were performed. The results are presented in Table 3.4. No significant changes in the amounts of secondary structure elements α -helix, β -sheet, β -turn, and random coil are observed between the untreated PPIp (dispersed in water for 30 min) and the treated PPI at t0 and t24.

Table 3.4: Secondary structure components of PPIp, PPI t0 and t24, and PPICur t0 and t24 derived from CD.

Sample	Random Coil (%)	α -helix (%)	β -sheet (%)	β -turn (%)
PPIp	52.5 $\pm$ 2.5	5.0 $\pm$ 0	25.5 $\pm$ 1.5	16 $\pm$ 1.0
PPI t0	48.7 $\pm$ 0.47	5.0 $\pm$ 0	28 $\pm$ 0.82	17.7 $\pm$ 0.47
PPI t24	49 $\pm$ 1.42	5.0 $\pm$ 0.82	28.7 $\pm$ 1.25	16.7 $\pm$ 0.47
PPICur t0	56.3 $\pm$ 2.87	6.7 $\pm$ 0.47	21.7 $\pm$ 2.05	15 $\pm$ 0.82
PPICur t24	60.3 $\pm$ 4.5	7.3 $\pm$ 0.47	19.7 $\pm$ 3.09	13.3 $\pm$ 1.25

Upon Cur loading, the proportions of α -helix increase slightly from 5% to 7%, while those of β -sheet and β -turn decrease from 27% to 21% and from 17% to 14%, respectively. In contrast, the proportion of random coil increases more substantially from 50% to 58% compared to the pure protein aggregates. The change in the secondary structure distribution suggests that β -structures become more disordered, and minor parts refold into α -helices upon the binding of Cur. Ren *et al.* treated a commercial pea protein isolate with a modified pH shifting method and studied the binding of Cur using CD. They observed an increase in the proportion of α -helix content and a decrease in the proportion of β -sheet content. The redistribution in secondary structure was attributed to hydrophobic interactions and hydrogen bond formation between the pea protein and Cur⁹⁵. Zhi *et al.* investigated the impact of pH shifting, heating, and ultrasound on the techno-functional properties of a commercial pea protein isolate, observing significant changes in the protein's secondary structure following these treatments¹⁴². Comparing PPICur t0 and t24, no significant difference was observed. It can thus be deduced that the precipitation of Cur cannot be attributed to a reorganization of the protein structure. The level of denaturation remains high throughout the entire preparation process, as indicated by a low content of α -helix¹⁴³. Therefore, the commonly used terms “unfolding” and “refolding” might not be suitable for describing the effect of pH shifting on protein structure. A more accurate description could be disrupting existing aggregates through electrostatic repulsion and reorganizing aggregates when decreasing ionic interactions. This also implies that the system is not dynamic, making the term “nanoaggregate” more precise than the commonly used term ‘molten globule’^{66,131,142}. Additionally, it is important to emphasize (again here) that the terms unfolding, refolding, and molten globule are closely linked to native protein structures, which are rarely encountered when working with commercially produced, impure plant protein isolates. Summarizing the CD analysis, various trends are reported in the literature. CD data and explanations derived therefrom are highly dependent on the measuring conditions, the pea protein product studied, the

preparation, and its denaturation state. Additionally, varying molar masses due to uncertainty with commercial products significantly affect the fit results. Therefore, data within one study can be compared, but absolute figures might vary depending on the product studied.

This study demonstrated that Cur binds to large, inert, soluble nanoaggregates formed through pH shifting and heating, without any preference for specific proteins. Cur is solubilized in a supersaturated state, and 96% precipitates again under constant stirring within 24 hours, resulting in a stable solution of PPICur t24. Cur loading and precipitation do not affect the surface charge of the carrier. Overall, 17% of PPIp and 10% of the protein precipitate alongside Cur. Since the experiments ruled out precipitation caused by major changes in the carrier, a closer look at Cur's role will be taken in the next section.

3.4.3. Binding Mechanism of PPICur

To study the binding between Cur and PPI(p), XRD, UV-Vis spectroscopy, and fluorescence spectroscopy were performed. The XRD patterns of PPICur t0 and t24, PPIp, and Cur are shown in Figure 3.8 **A**. Cur exhibits a characteristic pattern with sharp reflexes, indicating its crystallinity. The diffractogram of PPIp shows broad humps instead of sharp reflexes, suggesting its amorphous nature. In the diffractograms of PPICur t0 and t24, both patterns are observed. The reflexes at 31.7° , 45.46° , 56.45° , 66.29° , 75.29° , and 84.08° are attributed to NaCl as a result of the pH shifting^{96,144}. In the literature, the lack of Cur-related reflexes in the diffractograms of protein-curcumin complexes has been attributed to the amorphous binding of Cur to the protein^{95,96,99,107,121,123}.

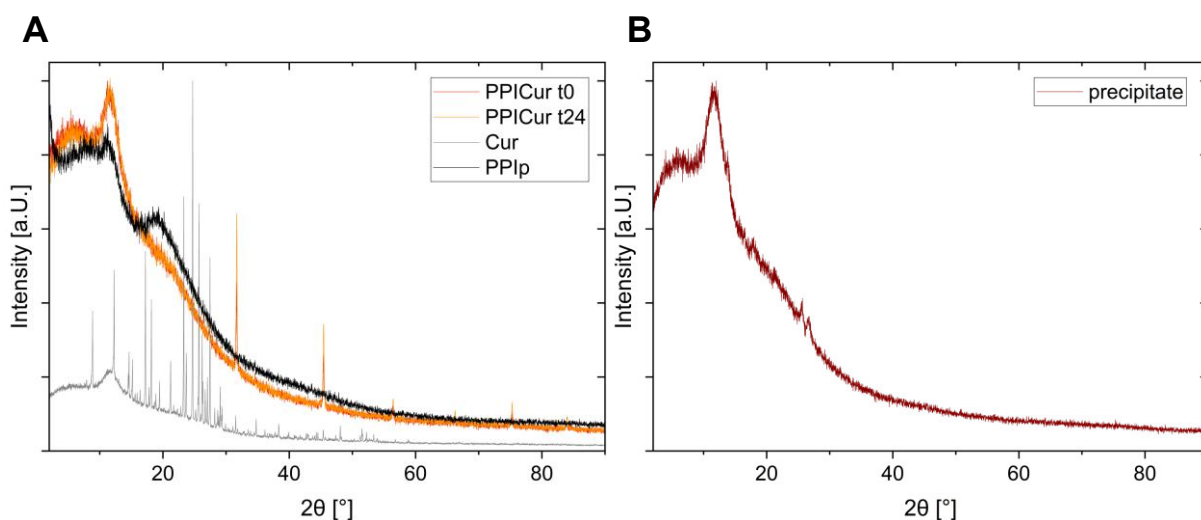


Figure 3.8: XRD patterns of PPICur t0 and t24, Cur, PPIp (**A**), and the precipitate (**B**).

Because of concentration differences, especially at t24, between plant protein and Cur, the informative value of the missing Cur reflexes is questionable in this case. It is more instructive to compare the supersaturated state at t0 with the stable state at t24, which do not differ. The lack of crystallinity at

t0 shows that the supersaturated state is not caused by crystalline Cur, but instead that the structural order of Cur has fully transformed to an amorphous structure. The precipitated Cur is also in an amorphous state (see Figure 3.8 B), likely due to rapid precipitation within 24 hours. The increased solubility of amorphous Cur in water is described in the literature¹⁴⁵. However, an increase in Cur solubility at t0 caused by the generation of unstable amorphous Cur could be excluded, as pH shifting of Cur in pure water resulted in only a minor amount of soluble Cur due to insolubility and degradation (see Figure SI 3 9).

UV-Vis spectra of PPICur were recorded using PPI as the dilution medium and a blank to assess the absorption properties (see Figure 3.9). The UV-Vis spectrum of PPICur t0 displays a characteristic profile with a peak maximum at 428 nm and a shoulder at 449 nm. The UV-Vis spectrum of PPICur t24 displays a less defined profile with a peak maximum at 410 nm and a significantly lower absorbance due to the precipitation of 96% of Cur (see Figure 3.9 A and B). HPLC analysis ruled out absorption changes due to degradation and confirmed the presence and stability of Cur in both samples. Fluorescence measurements were performed at the same absolute Cur concentration. The proportion of organic solvent in the pure Cur sample was below 1‰. Cur is a weak fluorophore in water¹⁴⁶, exhibiting a maximum emission around 550 nm and maximum excitation at 430 nm with an emerging shoulder around 350 nm at lower emission wavelengths (see Figure 3.10 A). The UV-Vis spectrum of Cur at a concentration of 5 μ M exhibits a maximum at 430 nm and an upstream shoulder around 355 nm (see Figure 3.9 D). Some instrumental noise from an aged deuterium lamp is visible in the range of 225 to 325 nm. The emission and excitation spectra of PPICur t0 and t24 exhibit peak maxima at 502 nm and 430 nm, respectively, regardless of the excitation or emission wavelengths (see Figure 3.10 B and C). The fluorescence intensity of Cur at t0 in the emission spectra is by a factor of 2.2 higher than that at t24. The Cur fluorescence intensity of the excitation spectra is higher by a factor of 1.8.

To understand the different absorption behaviors, the molecular structure of Cur and its behavior in solution are examined more closely. Cur's molecular structure is sensitive to the surrounding medium, as its absorption properties vary with the degree of protonation, which is influenced by pH and keto-enol tautomerism. In the fully protonated state, the equilibrium was reported to be strongly shifted towards the *cis*-keto-enol tautomer in all analyzed organic solvents, due to intramolecular hydrogen bonds^{147–150}. Cur spans a maximum absorption range of 408 to 440 nm, displaying a negative solvatochromic effect in non-polar solvents and a positive solvatochromic effect in polar solvents. The fluorescence emission spectra behave similarly and exhibit blue-shifted emission maxima for non-polar solvents and red-shifted maxima for polar ones^{20,149,150}. With increasing water content in an aqueous mixture of EtOH, a shift of the equilibrium from the keto-enol form towards the diketo form was reported in the literature^{151,152}. To monitor keto-enol tautomerism at room temperature, UV-Vis

spectroscopy was preferred, as it is rapid on the NMR timescale. The increasing presence of the diketo form is indicated by a rising shoulder at 355 nm¹⁵². Keto-enol tautomerism varies with temperature, favoring the diketo form at higher temperatures. The fluorescence emission is blue-shifted as the amount of the diketo form increases¹⁵³. Besides the positions of the absorption maxima, the various profiles of the UV-Vis spectra are also addressed in the literature. In polar solvents, a broad absorption band of Cur is typically observed, whereas in less polar solvents, fine structuring and shoulders emerge^{147,149,150}.

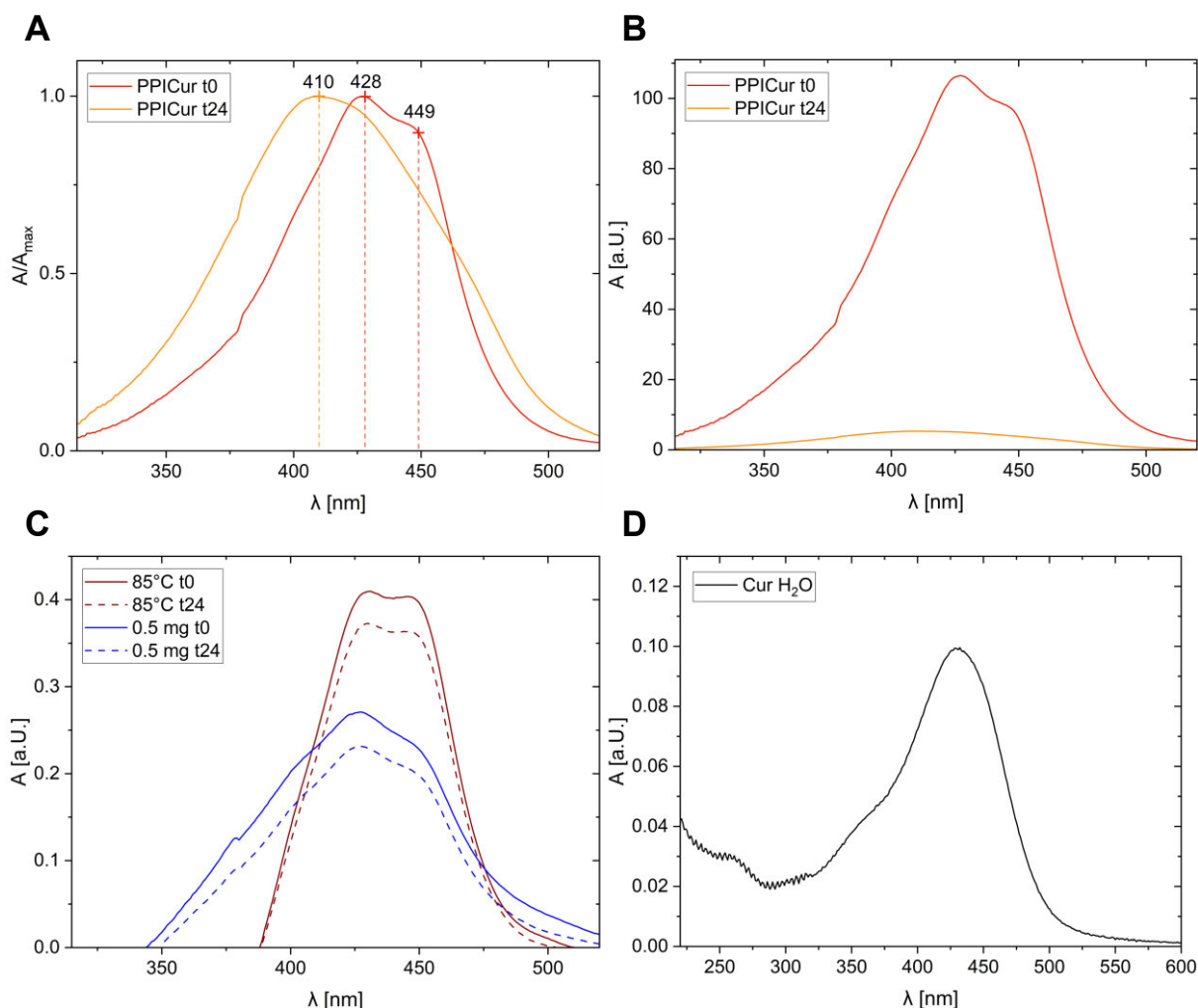


Figure 3.9: UV-Vis spectra of PPICur t0 and t24 with normalized (A) and concentration corrected (B) absorptions, prepared without pH shifting (C, brown), and without saturation (C, blue). UV-Vis spectrum of Cur in H₂O at a concentration of 5 μ M (D).

Chignell *et al.* and Khopde *et al.* noted some structuring, similar to that of PPICur t0, in the UV-Vis spectra of Cur dissolved in cyclohexane, benzene, toluene, and aqueous Triton X solution, which they attributed to the presence of different keto-enol isomers. The fluorescence spectra provided no evidence of coexisting isomers, indicating that all excited isomers have similar fluorescence properties or that all isomers produce the same excited state^{147,148}. This situation was also noted in the fluorescence measurements of PPICur t0 and t24 shown in Figure 3.10. Nardo *et al.* performed the

same spectroscopic experiments as Chignell *et al.* and Khopde *et al.*, dissolving Cur in various solvents and presenting the *cis* keto-enol form as the predominant form. However, they suggested that pronounced fine structuring may also indicate the presence of the *trans* diketo form, referring to the fine structuring of Cur in non-polar solvents observed by Chignell *et al.* (with the absorption maximum in cyclohexane at 408 nm and a distinct shoulder at 430 nm as a specific example). With increasing polarity of the solvent, the equilibrium shifted towards the *cis* keto-enol form, and the absorption maximum was red-shifted. They further considered that the *trans* keto-enol form might be favored in polar and H-bond donating solvents, as they disturb the intramolecular hydrogen bond that stabilizes the *cis* enol-isomer. This could explain the shoulders observed in polar and protic solvents¹⁴⁹. Since this is not seen in all protic solvents, and shoulders are present in polar, aprotic solvents such as DMSO, the counter opinion is that the *cis* keto-enol form always dominates, as discussed in the preceding part of the UV-Vis and fluorescence section. Regarding Cur in DMSO, Nardo *et al.* attributed an observed shoulder in the fluorescence emission spectrum to the coexistence of the *cis* keto-enol (at 471 nm) and the *trans* diketo form (at 502 nm)¹⁴⁹.

These considerations focus on the spectral behavior of Cur in organic solvents. Moving to aqueous systems, the solubilization of Cur using proteins and surfactants has already been examined in the literature. Adhikary *et al.* studied Curcumin in non-ionic, cationic, and anionic micelles. They observed an absorption peak maximum around 423-432 nm for all surfactants and a shoulder around 442-445 nm in dodecyl trimethylammonium bromide (DTAB) and Triton X solutions. They attributed the shoulder to vibronic transitions and compared it with the absorption spectra of Cur in toluene and chloroform, concluding that the environments of Cur in DTAB and Triton X micelles are similar to those in nonpolar, aprotic solvents. The shoulder was absent for Cur in the SDS solution, which was explained by the strong interaction between Cur and water in the Stern layer of the micelle. The fluorescence emission maxima were at 498 nm for DTAB, 501 nm for Triton X, and 548 nm for SDS. The positions of the maxima were explained by different levels of interaction between Cur and water, leading to the conclusion that Cur is located inside the micelle (for Triton X and DTAB) or near the outside (for SDS). Their investigations into excited-state intramolecular hydrogen atom transfer and the fluorescence upconversion of Cur in these systems led to the conclusion that a significant number of hydrogen bonds and water must be present in the Triton X micelles. Therefore, the environment around Cur in the micelle is rather hydrophilic than hydrophobic^{148,154}. No results were found that indicate the presence of multiple tautomers in micelles. Dutta *et al.* examined Cur in the presence of various anionic surfactants, both below and above the CMC. For $c > \text{CMC}$, only the enol form appeared to be present, whereas for $c < \text{CMC}$, they observed significant amounts of both tautomers¹⁵⁵.

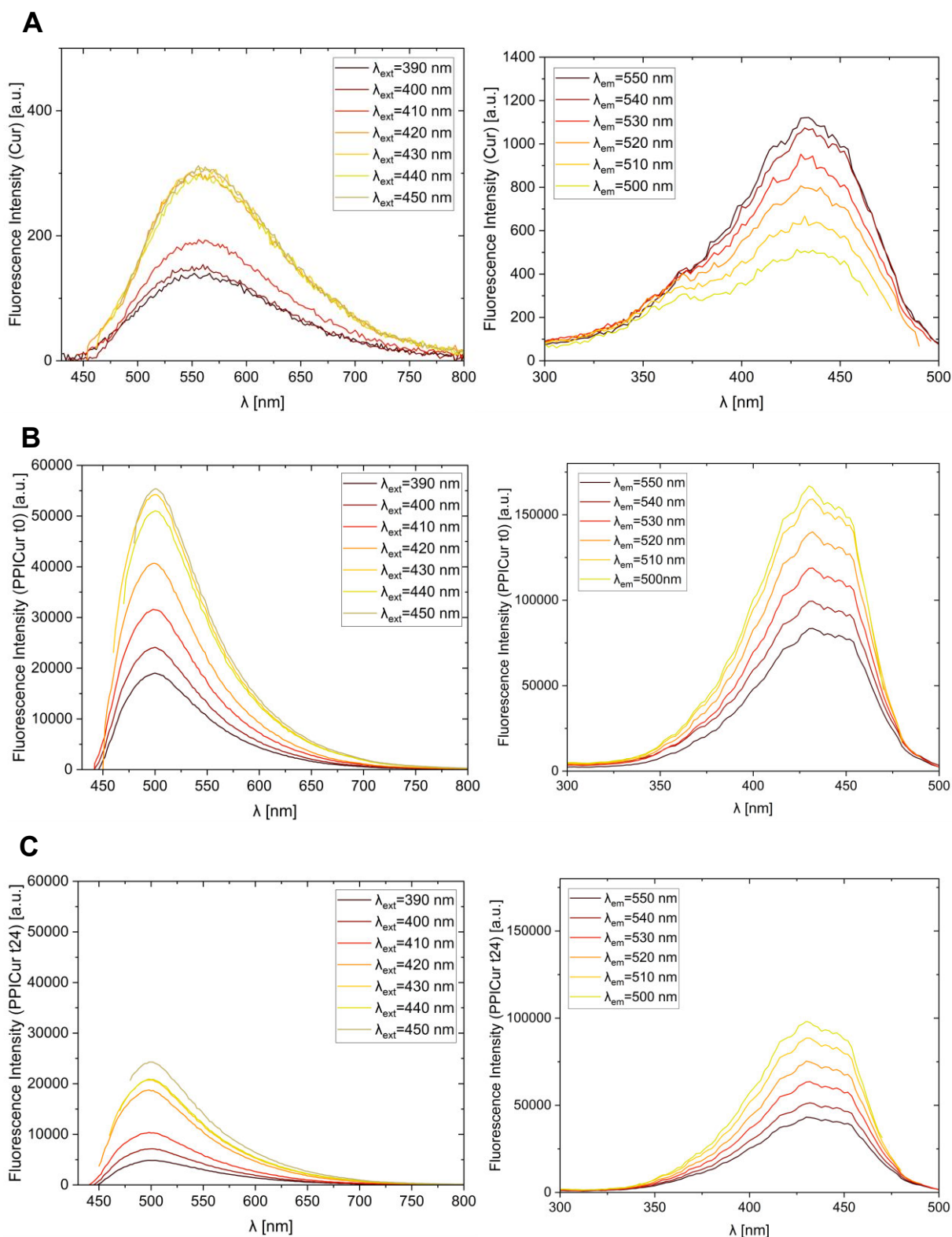


Figure 3.10: Fluorescence excitation and emission spectra of Cur in water (A), PPICur t0 (B), and PPICur t24 (C). The emission spectra (left) were measured with excitation wavelengths ranging from 390 to 450 nm. The excitation spectra (right) were measured with emission wavelengths between 500 nm and 550 nm.

The interactions between Cur and proteins were primarily studied for casein and serum albumins rather than for plant proteins, mainly due to the commercial production of plant proteins as impure powders and the lack of characterization. The main finding was the non-covalent binding to

hydrophobic binding sites of the proteins, as discussed in section 3.2.2.2. The UV-Vis spectra exhibited absorbance maxima between 425 nm and 440 nm, predominantly displaying structuring, while the fluorescence emission spectra demonstrated a blueshift to 500-510 nm in comparison to the maximum at 550 nm observed for Cur in water. In the reviewed literature, no information was found regarding the equilibrium of keto-enol tautomerism upon binding to (plant) proteins^{46,90,91,95,97,99,108,121,146,156–159}.

Returning to the experiments conducted with PPICur, different spectral behaviors were observed depending on the amount of loaded Cur and the preparation of PPI (see Figure 3.9 C). When PPI was not loaded with 8 mg/mL of Cur but only with 0.05 mg/mL of Cur (corresponding to Cur's solubility in PPICur t24), the maximum at 428 nm and the shoulder at 449 nm, along with a prominent shoulder at 410 nm, were visible. When PPI was prepared by heating only and loaded with Cur ($\beta_{\text{load}}=8$ mg/mL) without pH shifting, the absorption pattern showed the peak at 428 nm and the shoulder at 449 nm, while the shoulder at 410 nm was absent. In both cases, the overall shape of the absorption spectra remained unchanged, although a small amount of Cur still precipitated over 24 hours.

The considerations outlined above can be summarized as follows: the aqueous solubility of the very poorly soluble Cur can be increased through protein binding, facilitated by hydrogen bonding and hydrophobic interactions at hydrophobic binding sites within the proteins. In the present study, a supersaturation of Cur is generated using a pH shifting and heating method, and 96% of the solubilized Cur precipitates again within 24 hours. The UV-Vis spectra of PPICur t0 and t24 differ significantly in intensity, form, and the position of the absorption maxima. When changing the parameters during preparation (loading of Cur and preparation of PPI), supersaturation is nearly suppressed, and the spectral behavior is altered. No difference was observed in the positions of the peak maxima of fluorescence excitation and emission spectra of PPICur t0 and t24. Cur undergoes keto-enol tautomerism and exhibits a solvatochromic effect, depending on the polarity of the solvent.

Based on the results presented above and the available literature, two scenarios for Cur binding to PPI are possible. Cur and the protein isolate interact at a highly alkaline pH. In this condition, Cur is deprotonated, and the protein aggregate structure is disrupted. Therefore, no inside or outside of the nanoparticle exists during preparation, and Cur is randomly bound to all accessible binding sites. When the pH is readjusted to 7, the protein isolate forms more compact nanoaggregates, and Cur is found in different locations, inside and on the surface of the nanoaggregates. This results in both internal and external binding to the protein isolate aggregates, with a predominantly protein environment surrounding the Cur molecule in the first case and increased contact with the surrounding bulk water in the latter case. This has already been suggested in the literature but has not yet been experimentally demonstrated^{46,112,160}.

The UV-Vis spectra of PPICur show that the surface of PPI is initially supersaturated with Cur molecules at t_0 . The characteristic absorption pattern at t_0 indicates a more polar environment, suggesting contact with water and hydrogen bonding. Following the explanations of binding in micelles¹⁵⁴, external binding of Cur is suggested. Therefore, the precipitation of Cur might result from the too-tight packing of Cur molecules on the nanoparticle surface. Following the theory of nucleation, they aggregate and precipitate over time. The process is accelerated by stirring. The absorbance maximum of PPICur t_{24} shifts to 410 nm because the Cur molecules bound within the aggregate are in a nonpolar environment, do not precipitate, and become more prominent after the precipitation of the externally bound Cur molecules. Since not all of the latter precipitate, their contributions at 428 nm and 449 nm remain visible as shoulders. For the UV-Vis spectrum, it is reasonable to assume that the absorption at 410 nm is also present at t_0 but is masked by the much stronger absorption at 428 nm. The UV-Vis spectra of the two preparation modifications (loading with a small amount of Cur and preparation without pH shifting, see Figure 3.9 C) support this idea: using a relatively low amount of Cur (0.05 mg/mL instead of 8 mg/mL) to load PPI prevents the nanoparticle surface from becoming supersaturated to such an extent, and the contributions of all binding environments to the absorption are visible at t_0 . As a result, the aggregation of external Cur molecules is reduced, and only a small quantity of Cur precipitates over time. When PPICur is prepared by heating alone, the protein isolate aggregates are not broken up, so no internal binding should occur. This is reflected in the corresponding UV-Vis spectra at t_0 and t_{24} , which show only the pattern at 428 nm and 449 nm, but no peak or shoulder at 410 nm (see Figure 3.9 C). The constant positions of the fluorescence excitation and emission maxima at t_0 and t_{24} do not support this hypothesis. If Cur is exposed to different polarities and does not undergo keto-enol tautomerism or isomerization, its fluorescence behavior should also change. Tapal and Tiku also noted this when investigating SPI-Cur complexes⁹¹. This situation, however, does not necessarily weaken the proposed binding scenario, as water quenches Cur's fluorescence¹⁴⁶. The quantum yield of externally bound Cur could be lowered, making external binding undetectable. In addition, steady-state fluorescence averages over different states, preventing the distinction between multiple binding states. Although Cur concentration is constant in measurements at t_0 and t_{24} , emission at t_0 is 2.2 times higher, indicating possible rearrangement of Cur and static fluorescence quenching via stacking of Cur molecules or their interaction with amino acids. An inner-filter effect might also lower fluorescence at t_{24} . The current measurements align with the consensus in the literature that Cur is bound to the carrier; however, they neither support nor contradict the idea of internal and external binding.

The second scenario involves keto-enol tautomerism. The red-shifted shoulder of the peak at 428 nm could be due to either the presence of different enol isomers or a vibrational transition stabilized by hydrogen bonding. Because of the rapid readjustment to pH 7, reprotonated enolate molecules do not

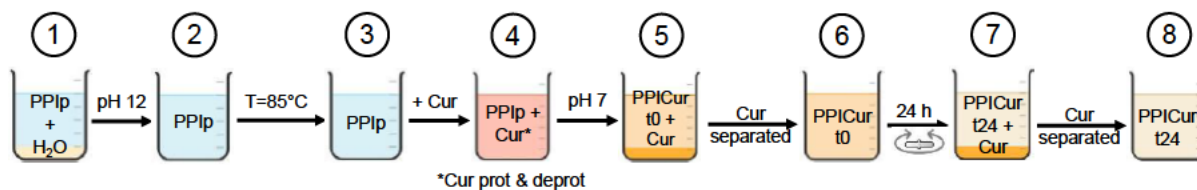
have enough time to adapt to the environment and form the enol tautomer with different isomers. Molecules bound close to the surface isomerize over time towards the diketo form, which is favored at higher water content. Since this tautomer is more prone to aggregation and the surface remains supersaturated, externally bound Cur precipitates over time, facilitated by energy input from stirring. After 24 hours, the equilibrium has shifted towards the diketo form, as shown by the emerging peak at 410 nm. Several different microenvironments exist in the aggregate, stabilizing both diketo and enol isomers, so no straightforward assignment can be made. The fluorescence data might not resolve the isomer mixture and instead indicate only an overall blueshift in the emission spectrum compared to Cur in water, suggesting Cur binding to the aggregate. A concentration-dependent adsorption behavior of Cur to fumed silica nanoparticles was reported by Kazakova *et al.* using UV-Vis spectroscopy and quantum chemical calculations for analysis. At low Cur concentrations, the enol tautomer adsorbed as a monolayer on the silica nanoparticle surface. At high Cur concentrations, aggregates of the keto form adsorbed as a bilayer¹⁵¹. These findings support the idea of a concentration-dependent isomerization of Cur. However, the diketo form lacks an extended electron system and does not absorb in the same spectral range as the enol form, unless the electron system is somehow extended. The findings from the two modified preparations do not support the second scenario either.

3.4.4. All Put Together: Mechanism Elucidation

Using current literature and experimental results from this study, a schematic mechanism of the preparation of PPI and its interactions with Cur is illustrated in Figure 3.11. PPIp is dispersed in water at a concentration of 1% (w/v) (10 mg/mL). 3 mg/mL PPIp is solubilized, consisting of 2 mg/mL protein. Due to industrial extraction, the solubilized PPIp exists as aggregates composed of a mixture of denatured proteins and organic impurities (1). When adjusted to pH 12, the ionizable groups become charged, leading to electrostatic repulsion and subsequent aggregate disassembly (2). Heating increases surface hydrophobicity by exposing previously hidden hydrophobic groups to the surrounding environment (3). The high alkaline pH and heat treatment notably improve the solubility of PPIp and cause a more compact aggregate structure. When Cur is added above its solubilization limit in alkaline water, Cur exists in equilibrium between deprotonated and protonated species. Through hydrophobic and electrostatic interactions, Cur interacts with the detangled protein aggregates (4). When readjusting to pH 7, Cur and the ionizable groups of the protein are re-protonated. Cur molecules that could not bind to the protein precipitate and the electrostatic repulsion within the aggregates decreases. Cur is bound internally and externally to PPI, with the particle surface being highly supersaturated (5). The precipitated Cur is separated from PPICur t0 via centrifugation and filtration (6). Within 24 hours, 96% of the Cur bound to the PPI surface precipitates

due to aggregation of the tightly bound Cur molecules or isomerization thereof. The involvement of PPI in this process was ruled out (7). The precipitated Cur is separated from PPICur t24 (8).

Macroscale:



Nanoscale:

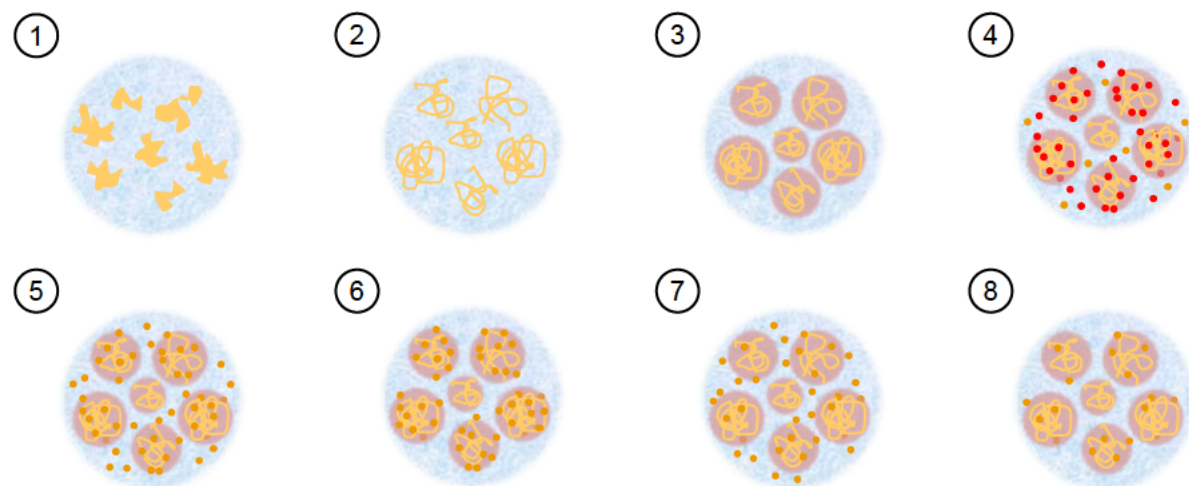


Figure 3.11: Schematic mechanism of the preparation, binding, and release of Cur with PPI. Preparation steps are shown on a macroscale above, while the processes occurring on the nanoscale are depicted below.

PPIp experienced significant modifications during the pH shifting and heating process. The relaxation of supersaturation could not be attributed to changes in the internal structure of the carrier, breakdown of its structure, presence of crystalline Cur species, or compound degradation. The precipitation of Cur was solely attributed to polyphenol aggregation or isomerization. However, minimal changes in the distribution of aggregates, as determined by SEC (see Figure 3.6 A), could trigger spontaneous precipitation.

In the investigation of PPICur and the elucidation of its binding mechanism, a deliberate decision was made not to focus solely on protein binding of Cur, considering that the aggregates formed mainly consist of pea protein, but also include a specific proportion of non-protein organic substances (see Figure 3.7 and Chapter 2 Table 2.2). Pan *et al.* reported the solubilization of Cur through self-assembled soybean polysaccharide nanoparticles¹⁶¹. Accordingly, the participation of organic matter in the solubilization of Cur cannot be ruled out. The proposed mechanism should generally apply to legume proteins, as they share the same storage protein composition¹³¹. Additionally, it is suggested to apply to other plant protein classes, provided that the processing parameters are tailored based on the heat denaturation temperature, the critical gelling concentration, and the isoelectric points of the

significant protein fractions. Since extraction typically induces some degree of denaturation, other commercial plant protein isolates are also denatured to some extent¹¹⁶.

Applying external stress, such as changes in pH, temperature shifts, and shear stress, disrupts aggregates and partially restores functionality of these raw materials. Since polyphenols bind to a structure that is first disrupted and then reorganized, expecting exclusively internal (encapsulation⁴⁶) or external binding (complexation⁴⁶) is unreasonable. Instead, a combination of both seems inevitable. Exclusive external binding is only probable if the aggregated structure remains intact. However, the literature has not yet addressed the kinetic stability of plant protein-Cur complexes or aggregates.

3.4.5. Kinetic Profiles and Stabilization Approaches for PPICur t0

The stability of nutraceutical formulations is a crucial factor in assessing their suitability for use. Precipitation kinetics depend on stirring and temperature (see Figure 3.12 A). At 22 °C with constant stirring, 80% of $\beta_{t0}(\text{Cur})$ remains in solution for eight hours; within 24 hours, 96% precipitates (filled blue diamonds). When left unstirred, 80% of $\beta_{t0}(\text{Cur})$ stays in solution after 24 hours, and 70% after 48 hours (unfilled blue diamonds). Raising the temperature to 37 °C, shows the same trend but over a shorter period. In the stirred sample, 96% of $\beta_{t0}(\text{Cur})$ precipitates within half an hour, while in the non-stirred sample, the same proportion precipitates within an hour (represented by filled and unfilled red symbols, respectively). The stability of Cur at 37 °C was confirmed by HPLC analysis (see Figure SI 3 10). Changes in pH over time ranged from 1% to 3% (data not shown). Considering a potential use as a supplement, drug, or food dye, PPICur will be supplied in powdered form. Therefore, it was lyophilized, and the kinetic stability of redispersed powders was assessed. 80-90% of PPICur t0 can be re-dispersed (confirmed by UV-Vis spectroscopy and NTA, see Figure SI 3 11), and Cur precipitates faster than freshly prepared samples, following the same trend (purple and dark blue symbols). The accelerated precipitation may be linked to the drying method and could likely be improved through a faster process, such as spray-drying, along with optimization of parameters^{162–164}.

Based on the previous sections, the aggregation and precipitation of Cur were linked to either the emerging contact of densely packed Cur molecules on the surface of the carrier or the isomerization of Cur. Stirring and increasing the temperature could accelerate both processes. To maintain $\beta_{t0}(\text{Cur})$ in biorelevant systems, two different approaches were assessed. Diluting the sample minimizes contact between supersaturated nanoparticles. It was tested for fresh and redispersed samples (**B** and **C**, green symbols) at 37 °C with a 1:10 dilution factor. No change in the precipitation behavior is observed for the stirred and the redispersed samples. Precipitation is slowed down only in the freshly prepared, undisturbed diluted sample; after an hour, about 40% of $\beta_{t0}(\text{Cur})$ remains in solution, while in the other samples it is about 10% (**B**, unfilled green circles). The second approach to slow down precipitation involved adding an impurity. Heffernan *et al.* produced supersaturated solutions of Cur

in propane-2-ol and extended its nucleation by introducing structurally related compounds demethoxycurcumin and bisdemethoxycurcumin (BDMC). They explained the longer nucleation with DFT and metadynamics computations, showing stronger binding of Cur-BDMC than Cur-Cur¹⁶⁵. The addition of BDMC was tested on freshly prepared undiluted samples, resulting in a slower precipitation rate, with 30% of Cur remaining in solution after one hour (B, brown symbols). Nucleation is probably slowed down in the same ways as described by Heffernan *et al.* Additionally, BDMC can act as a stacking breaker, slowing down the accumulation of Cur on the supersaturated surface.

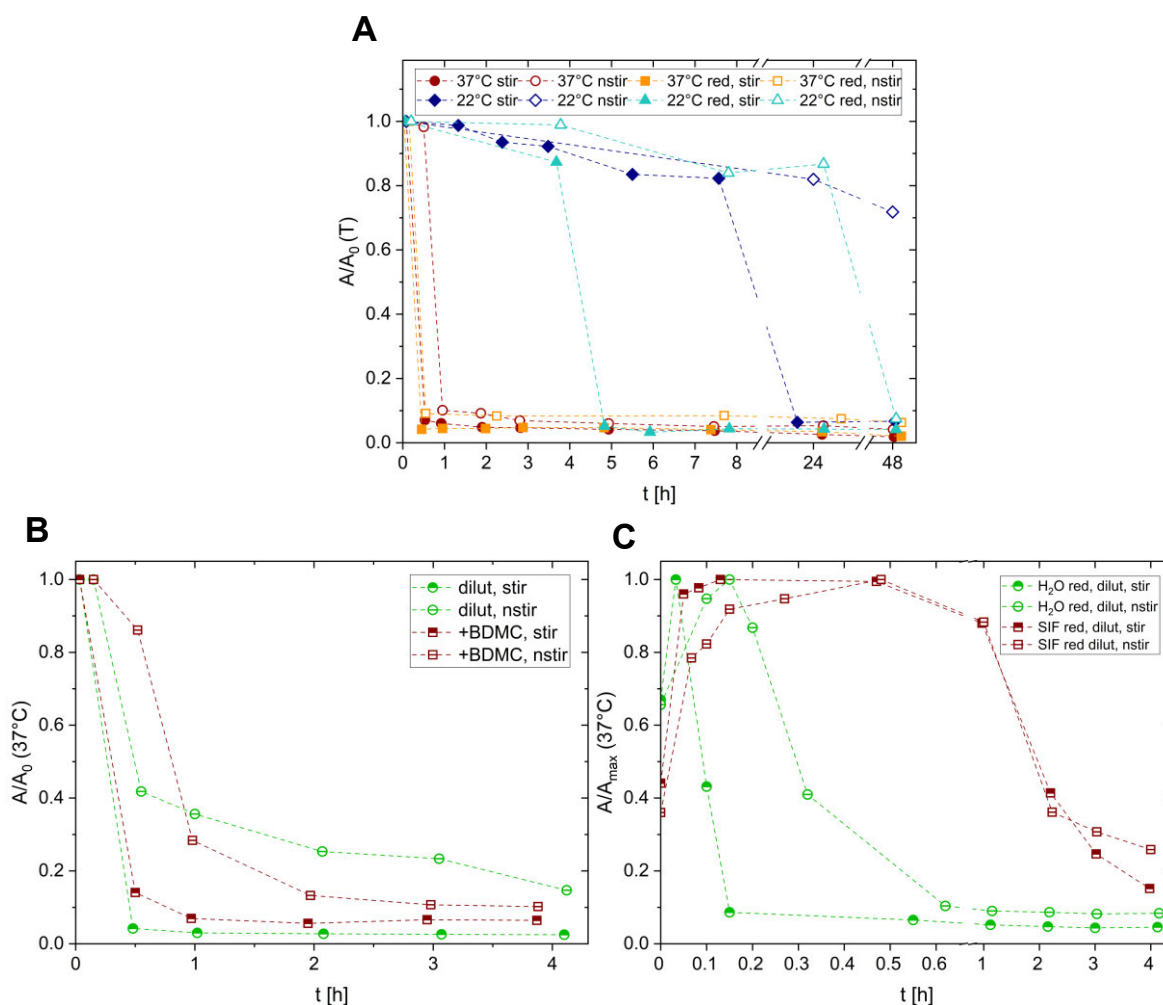


Figure 3.12: Kinetic profiles of PPICur t0 under various stirred and unstirred conditions: freshly prepared and redispersed PPICur t0 at 22°C and 37 °C (A), and diluted (1:10) and with the addition of bisdemethoxycurcumin (BDMC) (B). Release kinetics of redispersed lyophilized PPICur t0 in H_2O , diluted 1:10, and simulated intestinal fluid (SIF) (C).

Absorption of orally applied and protected Cur happens in the small intestine^{41,166}. Its release profile is evaluated in simulated intestinal fluid (SIF) (C, brown symbols). Unlike in the other experiments, absorption was measured at the point of redispersion rather than at complete dissolution of the powder. Dissolution and precipitation are significantly slowed in SIF compared to pure water, with 90% of $\beta_{10}(\text{Cur})$ in solution after one hour and 30% of $\beta_{10}(\text{Cur})$ after four hours in the unstirred sample. SIF contains bile salts, which form micelles that can either delay nucleation or incorporate the released Cur. The slower dissolution rate may result from competition between Cur loaded onto the protein

carrier and its incorporation into bile salt micelles^{167,168}. Considering all three effects together – dilution, a nature-identical mixture of curcuminoids, and the presence of naturally occurring solubilizers in the target – a notable slowdown in precipitation can be expected, leading to increased bioaccessibility and potential for uptake in the body. Whether the bioavailability, i.e., the proportion of the substance entering the bloodstream and accessible to organs and tissues¹⁶⁸, is improved, cannot be deduced from these data. Bioavailability tests require cell uptake and *in vivo* testing¹⁶⁹.

Switching from a stability to a pharmaceutical perspective, the precipitation profiles can also be viewed as release profiles. An apparent increase in solubility above the drug's equilibrium level, followed by rapid precipitation, is termed the spring effect. Extending the metastable solubility of a drug by adding precipitation inhibitors is known as the parachute effect. This release behavior is associated with supersaturated drug delivery systems (SDDSs)^{170–172}, and, based on the studies conducted, PPICur to can likely be classified as such. Another release behavior is the so-called burst release, which relates to the immediate separation of a drug from its carrier upon contact with the target medium¹⁷³. The literature has outlined various reasons for this behavior. Mehryar *et al.* attributed the observed burst release of Cur from sunflower protein isolate nanoparticles in PBS to a burst of the protein matrix and the detachment of Cur bound to the surface of the nanoparticle¹¹². They referred to Sadeghi *et al.*, who suggested a biphasic release pattern of Cur from albumin nanoparticles, resulting from internal (sustained release) and external (faster release) Cur binding¹⁶⁰. Teng *et al.* assigned the burst release from Cur-loaded soy protein nanoparticles to swelling and breakage of the protein matrix¹²¹. Rahman *et al.* and Jithan *et al.* described the burst release of Cur from solid lipid nanoparticles and albumin nanoparticles, respectively^{174,175}. Nevertheless, release behaviors of Cur-loaded carriers have also been documented, which do not conform to this pattern. Ye *et al.* monitored Cur release from kafirin and zein composite nanoparticles during simulated digestion and observed slow release in the saliva and gastric phases, with an increase in the intestinal phase after one hour¹⁷⁶. Mohammadian *et al.* reviewed the release of Cur from different protein complexes and suggested that the stability of the binding between Cur and its carrier, as well as its subsequent release, can vary depending on the different protein structures formed by various production methods⁴⁷. The release of Cur from carriers is mainly assessed after the solvent has been removed and the powder has been re-dispersed in a target medium. The kinetic stability of Cur binding before solvent removal is rarely analyzed. Consequently, Cur's release behavior has not yet been linked to its kinetic binding to the protein, although it is known that solvent and pH shifting methods induce supersaturation¹⁷². In summary, providing fundamental insights into how Cur binds to a modified pea protein isolate elucidated its supersaturation. Stabilization thereof is a highly studied yet challenging area in pharmaceutical science. The present study could contribute to the development of more effective stabilization strategies by emphasizing the importance of preparation and binding mechanisms.

3.5. Conclusion and Outlook

A comprehensive concept is introduced to understand how Cur binds to plant protein-based nanoparticles, particularly PPIp, prepared by a pH-driven method. It provides detailed insights into how the preparation method affects nanoparticle structure and subsequent binding of Cur, integrating existing literature and new experiments. SDS-PAGE and SEC data demonstrated the formation of large, soluble protein aggregates, whose size was decisive for Cur binding. Cur-loaded pea protein isolate nanoparticles PPICur were only kinetically stable, and Cur precipitated within 24 hours under constant stirring. The largest concentration gradient between t_0 and t_{24} and the highest concentration at time t_0 were observed for β (PPIp) at 1% (w/v), $\beta_{load}(\text{Cur})$ at 8 mg/mL, and heating PPIp before loading with Cur. The supersaturation and subsequent precipitation of Cur were studied using UV-Vis and fluorescence spectroscopy. It was associated either with the aggregation of tightly packed Cur molecules on the carrier surface or the isomerization and subsequent aggregation of Cur molecules. Changes or degradation of the protein carrier were largely ruled out, as shown by CD, particle size, and solubility measurements. The kinetics of Cur precipitation were examined under various conditions, showing accelerated precipitation when transitioning from room temperature to body temperature and stirring. Based on these findings, PPICur was proposed for classification as SDDS. Mechanism-related stabilization strategies were developed, resulting in a slowdown of precipitation by diluting PPICur and incorporating bisdemethoxycurcumin. By combining existing literature with supplementary experiments conducted in this study, substantial progress has been made in understanding plant protein carrier systems prepared using a pH-driven method and commercially available products, as well as their functionality in binding poorly soluble lipophilic compounds. Highlighting the kinetic and stable states of the formulation enables diverse applications in the food and pharmaceutical industries. A detailed understanding of molecular interactions helps both sectors optimize formulations in a targeted way, whether as a food colorant, nutraceutical, or drug. Revealing the kinetics of pH-driven protein-curcumin formulations could bridge an important gap between Cur's solubility and effective delivery in humans.

3.6. References

- (1) Nair, K. *Turmeric (Curcuma longa L.) and Ginger (Zingiber officinale Rosc.) - World's Invaluable Medicinal Spices: The Agronomy and Economy of Turmeric and Ginger*, **2019**. <https://doi.org/10.1007/978-3-030-29189-1>.
- (2) Beganovic, S.; Wittmann, C. Medical properties, market potential, and microbial production of golden polyketide curcumin for food, biomedical, and cosmetic applications. *Curr. Opin. Biotechnol.* **2024**, *87*, 103112-103126. <https://doi.org/10.1016/j.copbio.2024.103112>.
- (3) Peram, M. R.; Jalalpure, S. S.; Palkar, M. B.; Diwan, P. V. Stability studies of pure and mixture form of curcuminoids by reverse phase-HPLC method under various experimental stress conditions. *Food Sci. Biotechnol.* **2017**, *26*, 591–602. <https://doi.org/10.1007/s10068-017-0087-1>.
- (4) Kharat, M.; Du, Z.; Zhang, G.; McClements, D. J. Physical and Chemical Stability of Curcumin in Aqueous Solutions and Emulsions: Impact of pH, Temperature, and Molecular Environment. *J. Agric. Food Chem.* **2017**, *65*, 1525–1532. <https://doi.org/10.1021/acs.jafc.6b04815>.
- (5) Basnet, P.; Skalko-Basnet, N. Curcumin: an anti-inflammatory molecule from a curry spice on the path to cancer treatment. *Molecules* **2011**, *16*, 4567–4598. <https://doi.org/10.3390/molecules16064567>.
- (6) Niranjana, A.; Singh, S.; Dhiman, M.; Tewari, S. K. Biochemical Composition of Curcuma longa L. Accessions. *Anal. Lett.* **2013**, *46*, 1069–1083. <https://doi.org/10.1080/00032719.2012.751541>.
- (7) Tayyem, R. F.; Heath, D. D.; Al-Delaimy, W. K.; Rock, C. L. Curcumin content of turmeric and curry powders. *Nutr. Cancer* **2006**, *55*, 126–131. https://doi.org/10.1207/s15327914nc5502_2.
- (8) Ciuca, M. D.; Racovita, R. C. Curcumin: Overview of Extraction Methods, Health Benefits, and Encapsulation and Delivery Using Microemulsions and Nanoemulsions. *Int. J. Mol. Sci.* **2023**, *24*, 8874–8903. <https://doi.org/10.3390/ijms24108874>.
- (9) Jayaprakasha, G. K.; Jaganmohan Rao, L.; Sakariah, K. K. Antioxidant activities of curcumin, demethoxycurcumin and bisdemethoxycurcumin. *Food Chem.* **2006**, *98*, 720–724. <https://doi.org/10.1016/j.foodchem.2005.06.037>.
- (10) Yixuan, L.; Qaria, M. A.; Sivasamy, S.; Jianzhong, S.; Daochen, Z. Curcumin production and bioavailability: A comprehensive review of curcumin extraction, synthesis, biotransformation and delivery systems. *Ind. Crops Prod.* **2021**, *172*, 114050–114070. <https://doi.org/10.1016/j.indcrop.2021.114050>.
- (11) Pabst, G., Ed. *Köhler's Medizinal-Pflanzen in naturgetreuen Abbildungen mit kurz erläuterndem Texte. Atlas zur Pharmacopoea germanica, austriaca, belgica, danica, helvetica, hungarica, rossica, suecica, Neerlandica, British pharmacopoeia, zum Codex medicamentarius, sowie zur Pharmacopoeia of the United States of America 2*; Franz Eugen Köhler: Gera-Untermhaus, **1889**.

- (12) Manasa, P. S. L.; Kamble, A. D.; Chilakamarthi, U. Various Extraction Techniques of Curcumin—A Comprehensive Review. *ACS Omega* **2023**, *8*, 34868–34878. <https://doi.org/10.1021/acsomega.3c04205>.
- (13) Degot, P.; Huber, V.; Touraud, D.; Kunz, W. Curcumin extracts from *Curcuma Longa* – Improvement of concentration, purity, and stability in food-approved and water-soluble surfactant-free microemulsions. *Food Chem.* **2021**, *339*, 128140–128148. <https://doi.org/10.1016/j.foodchem.2020.128140>.
- (14) Degot, P.; Huber, V.; Hofmann, E.; Hahn, M.; Touraud, D.; Kunz, W. Solubilization and extraction of curcumin from *Curcuma Longa* using green, sustainable, and food-approved surfactant-free microemulsions. *Food Chem.* **2021**, *336*, 127660–127668. <https://doi.org/10.1016/j.foodchem.2020.127660>.
- (15) Huber, V.; Muller, L.; Degot, P.; Touraud, D.; Kunz, W. NADES-based surfactant-free microemulsions for solubilization and extraction of curcumin from *Curcuma Longa*. *Food Chem.* **2021**, *355*, 129624–129631. <https://doi.org/10.1016/j.foodchem.2021.129624>.
- (16) Huber, V.; Muller, L.; Hioe, J.; Degot, P.; Touraud, D.; Kunz, W. Improvement of the Solubilization and Extraction of Curcumin in an Edible Ternary Solvent Mixture. *Molecules* **2021**, *26*, 7702–7721. <https://doi.org/10.3390/molecules26247702>.
- (17) Nelson, K. M.; Dahlin, J. L.; Bisson, J.; Graham, J.; Pauli, G. F.; Walters, M. A. The Essential Medicinal Chemistry of Curcumin. *J. Med. Chem.* **2017**, *60*, 1620–1637. <https://doi.org/10.1021/acs.jmedchem.6b00975>.
- (18) Heger, M.; van Golen, R. F.; Broekgaarden, M.; Michel, M. C. The molecular basis for the pharmacokinetics and pharmacodynamics of curcumin and its metabolites in relation to cancer. *Pharmacol. Rev.* **2014**, *66*, 222–307. <https://doi.org/10.1124/pr.110.004044>.
- (19) Zheng, B.; McClements, D. J. Formulation of More Efficacious Curcumin Delivery Systems Using Colloid Science: Enhanced Solubility, Stability, and Bioavailability. *Molecules* **2020**, *25*, 2791–2816. <https://doi.org/10.3390/molecules25122791>.
- (20) Priyadarsini, K. I. Photophysics, photochemistry and photobiology of curcumin: Studies from organic solutions, bio-mimetics and living cells. *J. Photochem. Photobiol. C: Photochem. Rev.* **2009**, *10*, 81–95. <https://doi.org/10.1016/j.jphotochemrev.2009.05.001>.
- (21) Bayach, I.; Sancho-García, J. C.; Di Meo, F.; Weber, J.-F.; Trouillas, P. π -Stacked polyphenolic dimers: A case study using dispersion-corrected methods. *Chem. Phys. Lett.* **2013**, *578*, 120–125. <https://doi.org/10.1016/j.cplett.2013.05.064>.
- (22) Santos, J. R. C.; Abreu, P. E.; Marques, J. M. C. Aggregation patterns of curcumin and piperine mixtures in different polar media. *Phys. Chem. Chem. Phys.* **2023**, *25*, 19899–19910. <https://doi.org/10.1039/D3CP00096F>.
- (23) Food and Drug Administration Office of Food Additive Safety Agency. *GRAS Notices No. Grn 000460*, **2013**. <https://hfppappexternal.fda.gov/scripts/fdcc/index.cfm?set=grasnotices&id=460> (accessed Dec 4, 2025).
- (24) Amchova, P.; Kotolova, H.; Ruda-Kucerova, J. Health safety issues of synthetic food colorants. *Regul. Toxicol. Pharmacol.* **2015**, *73*, 914–922. <https://doi.org/10.1016/j.yrtph.2015.09.026>.

- (25) Brower, V. Nutraceuticals: poised for a healthy slice of the healthcare market? *Nat. Biotechnol.* **1998**, *16*, 728–731. <https://doi.org/10.1038/nbt0898-728>.
- (26) Karłowicz-Bodalska, K.; Han, S.; Freier, J.; Smolenski, M.; Bodalska, A. Curcuma longa as medicinal herb in the treatment of diabetic complications. *Acta Pol. Pharm.* **2017**, *74*, 605–610.
- (27) Chin, D.; Huebbe, P.; Pallauf, K.; Rimbach, G. Neuroprotective properties of curcumin in Alzheimer's disease—merits and limitations. *Curr. Med. Chem.* **2013**, *20*, 3955–3985. <https://doi.org/10.2174/09298673113209990210>.
- (28) Willenbacher, E.; Khan, S. Z.; Mujica, S. C. A.; Trapani, D.; Hussain, S.; Wolf, D.; Willenbacher, W.; Spizzo, G.; Seeber, A. Curcumin: New Insights into an Ancient Ingredient against Cancer. *Int. J. Mol. Sci.* **2019**, *20*, 1808–1821. <https://doi.org/10.3390/ijms20081808>.
- (29) Bisson, J.; McAlpine, J. B.; Friesen, J. B.; Chen, S.-N.; Graham, J.; Pauli, G. F. Can Invalid Bioactives Undermine Natural Product-Based Drug Discovery? *J. Med. Chem.* **2016**, *59*, 1671–1690. <https://doi.org/10.1021/acs.jmedchem.5b01009>.
- (30) Brahmachari, V.; Jain, S. Pharmacokinetics and Pharmacodynamics. In *Encyclopedia of Systems Biology*; Dubitzky, W., Wolkenhauer, O., Cho, K.-H., Yokota, H., Eds.; Springer: New York, **2013**; pp 1687–1688. https://doi.org/10.1007/978-1-4419-9863-7_843.
- (31) Clairambault, J. Pharmacokinetics-Pharmacodynamics. In *Encyclopedia of Systems Biology*; Dubitzky, W., Wolkenhauer, O., Cho, K.-H., Yokota, H., Eds.; Springer: New York, **2013**; pp 1688–1689. https://doi.org/10.1007/978-1-4419-9863-7_696.
- (32) Lucas, A. J.; Sproston, J. L.; Barton, P.; Riley, R. J. Estimating human ADME properties, pharmacokinetic parameters and likely clinical dose in drug discovery. *Expert Opin. Drug Discov.* **2019**, *14*, 1313–1327. <https://doi.org/10.1080/17460441.2019.1660642>.
- (33) Lao, C. D.; 4th Ruffin, M. T.; Normolle, D.; Heath, D. D.; Murray, S. I.; Bailey, J. M.; Boggs, M. E.; Crowell, J.; Rock, C. L.; Brenner, D. E. Dose escalation of a curcuminoid formulation. *BMC complementary and alternative medicine* **2006**, *6*, 10–14. <https://doi.org/10.1186/1472-6882-6-10>.
- (34) Cheng, A. L.; Hsu, C. H.; Lin, J. K.; Hsu, M. M.; Ho, Y. F.; Shen, T. S.; Ko, J. Y.; Lin, J. T.; Lin, B. R.; Ming-Shiang, W.; *et al.* Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res.* **2001**, *21*, 2895–2900.
- (35) Baell, J. B. Feeling Nature???s PAINS: Natural Products, Natural Product Drugs, and Pan Assay Interference Compounds (PAINS). *J. Nat. Prod.* **2016**, *79*, 616–628. <https://doi.org/10.1021/acs.jnatprod.5b00947>.
- (36) Hegde, M.; Girisa, S.; BharathwajChetty, B.; Vishwa, R.; Kunnumakkara, A. B. Curcumin Formulations for Better Bioavailability: What We Learned from Clinical Trials Thus Far? *ACS Omega* **2023**, *8*, 10713–10746. <https://doi.org/10.1021/acsomega.2c07326>.
- (37) Tomeh, M. A.; Hadianamrei, R.; Zhao, X. A Review of Curcumin and Its Derivatives as Anticancer Agents. *Int. J. Mol. Sci.* **2019**, *20*, 1033–1059. <https://doi.org/10.3390/ijms20051033>.

- (38) Yuan, Y.; Ma, M.; Xu, Y.; Wang, D. Construction of biopolymer-based nanoencapsulation of functional food ingredients using the pH-driven method: a review. *Crit. Rev. Food Sci. Nutr.* **2023**, *63*, 5724–5738. <https://doi.org/10.1080/10408398.2021.2023858>.
- (39) Tabanelli, R.; Brogi, S.; Calderone, V. Improving Curcumin Bioavailability: Current Strategies and Future Perspectives. *Pharmaceutics* **2021**, *13*, 1715–1752. <https://doi.org/10.3390/pharmaceutics13101715>.
- (40) Yakubu, J.; Pandey, A. V. Innovative Delivery Systems for Curcumin: Exploring Nanosized and Conventional Formulations. *Pharmaceutics* **2024**, *16*, 637–655. <https://doi.org/10.3390/pharmaceutics16050637>.
- (41) Joye, I.; Davidov-Pardo, G.; McClements, D. J. Nanotechnology for increased micronutrient bioavailability. *Trends Food Sci. Technol.* **2015**, *40*, 168–182. <https://doi.org/10.1016/j.tifs.2014.08.006>.
- (42) Gomes, A.; do Amaral Sobral, P. J. Plant Protein-Based Delivery Systems: An Emerging Approach for Increasing the Efficacy of Lipophilic Bioactive Compounds. *Molecules* **2022**, *27*, 60–90. <https://doi.org/10.3390/molecules27010060>.
- (43) Wan, Z.-L.; Guo, J.; Yang, X.-Q. Plant protein-based delivery systems for bioactive ingredients in foods. *Food Funct.* **2015**, *6*, 2876–2889. <https://doi.org/10.1039/c5fo00050e>.
- (44) Tang, C.-H. Assembly of food proteins for nano- encapsulation and delivery of nutraceuticals (a mini-review). *Food Hydrocoll.* **2021**, *117*, 106710–106721. <https://doi.org/10.1016/j.foodhyd.2021.106710>.
- (45) Tang, C.-H. Strategies to utilize naturally occurring protein architectures as nanovehicles for hydrophobic nutraceuticals. *Food Hydrocoll.* **2021**, *112*, 106344–106364. <https://doi.org/10.1016/j.foodhyd.2020.106344>.
- (46) Tang, C.-H. Nanocomplexation of proteins with curcumin: From interaction to nanoencapsulation (A review). *Food Hydrocoll.* **2020**, *109*, 106106–106128. <https://doi.org/10.1016/j.foodhyd.2020.106106>.
- (47) Mohammadian, M.; Salami, M.; Assadpour, E.; Jafari, S. Curcumin-protein complexes: Technological and biological functionalities. *Trends Food Sci. Technol.* **2024**, *145*, 104372–104386. <https://doi.org/10.1016/j.tifs.2024.104372>.
- (48) Wang, X.; Gao, Y. Effects of length and unsaturation of the alkyl chain on the hydrophobic binding of curcumin with Tween micelles. *Food Chem.* **2018**, *246*, 242–248. <https://doi.org/10.1016/j.foodchem.2017.11.024>.
- (49) Mondal, S.; Moulik, S. Colloidal Dispersions of Lipids and Curcumin, and the Solubility and Degradation Kinetics of the Latter in Micellar Solution. *Soft Mater.* **2015**, *13*, 118–125. <https://doi.org/10.1080/1539445X.2015.1025981>.
- (50) Pan, K.; Zhong, Q.; Baek, S. J. Enhanced dispersibility and bioactivity of curcumin by encapsulation in casein nanocapsules. *J. Agric. Food Chem.* **2013**, *61*, 6036–6043. <https://doi.org/10.1021/jf400752a>.

- (51) Parikh, A.; Kathawala, K.; Song, Y.; Zhou, X.-F.; Garg, S. Curcumin-loaded self-nanomicellizing solid dispersion system: part I: development, optimization, characterization, and oral bioavailability. *Drug Deliv. Transl. Res.* **2018**, *8*, 1389–1405. <https://doi.org/10.1007/s13346-018-0543-3>.
- (52) Ma, Y.; Chen, S.; Liao, W.; Zhang, L.; Liu, J.; Gao, Y. Formation, Physicochemical Stability, and Redispersibility of Curcumin-Loaded Rhamnolipid Nanoparticles Using the pH-Driven Method. *J. Agric. Food Chem.* **2020**, *68*, 7103–7111. <https://doi.org/10.1021/acs.jafc.0c01326>.
- (53) Peng, S.; Li, Z.; Zou, L.; Liu, W.; Liu, C.; McClements, D. J. Enhancement of Curcumin Bioavailability by Encapsulation in Sophorolipid-Coated Nanoparticles: An in Vitro and in Vivo Study. *J. Agric. Food Chem.* **2018**, *66*, 1488–1497. <https://doi.org/10.1021/acs.jafc.7b05478>.
- (54) Yi, J.; Kang, L.; Luo, D.; Fan, Y. Enhanced solubility, stability, bioaccessibility, and antioxidant activity of curcumin with hydrolyzed pea protein-based nano-micelles: pH-driven method vs ethanol-induced method. *Int. J. Biol. Macromol.* **2025**, *291*, 139106–139116. <https://doi.org/10.1016/j.ijbiomac.2024.139106>.
- (55) Wang, S.; Ye, F.; Wei, F.; Zhao, G. Spray-drying of curcumin-loaded octenylsuccinated corn dextrin micelles stabilized with maltodextrin. *Powder Technol.* **2017**, *307*, 56–62. <https://doi.org/10.1016/j.powtec.2016.11.018>.
- (56) Stanciu, M. C.; Nichifor, M.; Mocanu, G.; Tuchilus, C.; Ailiesei, G. L. Block copolymers containing dextran and deoxycholic acid polyesters. Synthesis, self-assembly and hydrophobic drug encapsulation. *Carbohydr. Polym.* **2019**, *223*, 115118–115127. <https://doi.org/10.1016/j.carbpol.2019.115118>.
- (57) Zhi, K.; Wang, R.; Wei, J.; Shan, Z.; Shi, C.; Xia, X. Self-assembled micelles of dual-modified starch via hydroxypropylation and subsequent debranching with improved solubility and stability of curcumin. *Food Hydrocoll.* **2021**, *118*, 106809–106820. <https://doi.org/10.1016/j.foodhyd.2021.106809>.
- (58) Yu, H.; Huang, Q. Enhanced in vitro anti-cancer activity of curcumin encapsulated in hydrophobically modified starch. *Food Chem.* **2010**, *119*, 669–674. <https://doi.org/10.1016/j.foodchem.2009.07.018>.
- (59) Tønnesen, H. H.; Másson, M.; Loftsson, T. Studies of curcumin and curcuminoids. XXVII. Cyclodextrin complexation: solubility, chemical and photochemical stability. *Int. J. Pharm.* **2002**, *244*, 127–135. [https://doi.org/10.1016/s0378-5173\(02\)00323-x](https://doi.org/10.1016/s0378-5173(02)00323-x).
- (60) Yallapu, M. M.; Jaggi, M.; Chauhan, S. C. β -Cyclodextrin-curcumin self-assembly enhances curcumin delivery in prostate cancer cells. *Colloids Surf. B: Biointerfaces* **2010**, *79*, 113–125. <https://doi.org/10.1016/j.colsurfb.2010.03.039>.
- (61) Tomren, M. A.; Másson, M.; Loftsson, T.; Tønnesen, H. H. Studies on curcumin and curcuminoids: XXXI. Symmetric and asymmetric curcuminoids: Stability, activity and complexation with cyclodextrin. *Int. J. Pharm.* **2007**, *338*, 27–34. <https://doi.org/10.1016/j.ijpharm.2007.01.013>.

- (62) Al Fatease, A.; Shoman, M. E.; Abourehab, M. A. S.; Abou-Taleb, H. A.; Abdelkader, H. A Novel Curcumin Arginine Salt: A Solution for Poor Solubility and Potential Anticancer Activities. *Molecules* **2022**, *28*, 262–272. <https://doi.org/10.3390/molecules28010262>.
- (63) Tikekar, R. V.; Pan, Y.; Nitin, N. Fate of curcumin encapsulated in silica nanoparticle stabilized Pickering emulsion during storage and simulated digestion. *Food Res. Int.* **2013**, *51*, 370–377. <https://doi.org/10.1016/j.foodres.2012.12.027>.
- (64) Araiza-Calahorra, A.; Sarkar, A. Pickering emulsion stabilized by protein nanogel particles for delivery of curcumin: Effects of pH and ionic strength on curcumin retention. *Food Struct.* **2019**, *21*, 100113-100125. <https://doi.org/10.1016/j.foostr.2019.100113>.
- (65) Marefati, A.; Bertrand, M.; Sjöö, M.; Dejmek, P.; Rayner, M. Storage and digestion stability of encapsulated curcumin in emulsions based on starch granule Pickering stabilization. *Food Hydrocoll.* **2017**, *63*, 309–320. <https://doi.org/10.1016/j.foodhyd.2016.08.043>.
- (66) Qiao, X.; Liu, F.; Kong, Z.; Yang, Z.; Dai, L.; Wang, Y.; Sun, Q.; McClements, D. J.; Xu, X. Pickering emulsion gel stabilized by pea protein nanoparticle induced by heat-assisted pH-shifting for curcumin delivery. *J. Food Eng.* **2023**, *350*, 111504–111517. <https://doi.org/10.1016/j.jfoodeng.2023.111504>.
- (67) Ali, M.; Khan, N. R.; Subhan, Z.; Mehmood, S.; Amin, A.; Rabbani, I.; -Rehman, F.; Basit, H. M.; Syed, H. K.; Khan, I. U.; *et al.* Novel Curcumin-Encapsulated α -Tocopherol Nanoemulsion System and Its Potential Application for Wound Healing in Diabetic Animals. *BioMed Res. Int.* **2022**, *2022*, 7669255-7669271. <https://doi.org/10.1155/2022/7669255>.
- (68) Bergonzi, M. C.; Hamdouch, R.; Mazzacova, F.; Isacchi, B.; Bilia, A. R. Optimization, characterization and in vitro evaluation of curcumin microemulsions. *LWT* **2014**, *59*, 148–155. <https://doi.org/10.1016/j.lwt.2014.06.009>.
- (69) Hu, L.; Jia, Y.; Niu, F.; Jia, Z.; Yang, X.; Jiao, K. Preparation and enhancement of oral bioavailability of curcumin using microemulsions vehicle. *J. Aric. Food Chem.* **2012**, *60*, 7137–7141. <https://doi.org/10.1021/jf204078t>.
- (70) Zeeb, B.; Saberi, A. H.; Weiss, J.; McClements, D. J. Formation and characterization of filled hydrogel beads based on calcium alginate: Factors influencing nanoemulsion retention and release. *Food Hydrocoll.* **2015**, *50*, 27–36. <https://doi.org/10.1016/j.foodhyd.2015.02.041>.
- (71) Zhang, Z.; Zhang, R.; Zou, L.; Chen, L.; Ahmed, Y.; Al Bishri, W.; Balamash, K.; McClements, D. J. Encapsulation of curcumin in polysaccharide-based hydrogel beads: Impact of bead type on lipid digestion and curcumin bioaccessibility. *Food Hydrocoll.* **2016**, *58*, 160–170. <https://doi.org/10.1016/j.foodhyd.2016.02.036>.
- (72) Mohammadian, M.; Salami, M.; Momen, S.; Alavi, F.; Emam-Djomeh, Z. Fabrication of curcumin-loaded whey protein microgels: Structural properties, antioxidant activity, and in vitro release behavior. *LWT* **2018**, *103*, 94–100. <https://doi.org/10.1016/j.lwt.2018.12.076>.
- (73) Mangalathillam, S.; Rejinold, N. S.; Nair, A.; Lakshma nan, V.-K.; Nair, S. V.; Jayakumar, R. Curcumin loaded chitin nanogels for skin cancer treatment via the transdermal route. *Nanoscale* **2012**, *4*, 239–250. <https://doi.org/10.1039/c1nr11271f>.

- (74) Pancholi, V.; Smina, T. P.; Kunnumakkara, A. B.; Maliakel, B.; Krishnakumar, I. M. Safety assessment of a highly bioavailable curcumin-galactomannoside complex (CurQfen) in healthy volunteers, with a special reference to the recent hepatotoxic reports of curcumin supplements: A 90-days prospective study. *Toxicol. Rep.* **2021**, *8*, 1255–1264. <https://doi.org/10.1016/j.toxrep.2021.06.008>.
- (75) Peng, S.; Zou, L.; Zhou, W.; Liu, W.; Liu, C.; McClements, D. J. Encapsulation of Lipophilic Polyphenols into Nanoliposomes Using pH-Driven Method: Advantages and Disadvantages. *J. Agric. Food Chem.* **2019**, *67*, 7506–7511. <https://doi.org/10.1021/acs.jafc.9b01602>.
- (76) Chen, H.-W.; Chen, S.-D.; Wu, H.-T.; Cheng, C.-H.; Chiou, C.-S.; Chen, W.-T. Improvement in Curcumin's Stability and Release by Formulation in Flexible Nano-Liposomes. *Nanomaterials* **2024**, *14*, 1836–1851. <https://doi.org/10.3390/nano14221836>.
- (77) Li, R.; Deng, L.; Cai, Z.; Zhang, S.; Wang, K.; Li, L.; Ding, S.; Zhou, C. Liposomes coated with thiolated chitosan as drug carriers of curcumin. *Mater. Sci. Eng. C Mater. Biol. Appl.* **2017**, *80*, 156–164. <https://doi.org/10.1016/j.msec.2017.05.136>.
- (78) Chen, Y.; Wu, Q.; Zhang, Z.; Yuan, L.; Liu, X.; Zhou, L. Preparation of curcumin-loaded liposomes and evaluation of their skin permeation and pharmacodynamics. *Molecules* **2012**, *17*, 5972–5987. <https://doi.org/10.3390/molecules17055972>.
- (79) Bulboacă, A. E.; Bolboacă, S. D.; Stănescu, I. C.; Sfrângeu, C. A.; Porfire, A.; Tefas, L.; Bulboacă, A. C. The effect of intravenous administration of liposomal curcumin in addition to sumatriptan treatment in an experimental migraine model in rats. *Int. J. Nanomed.* **2018**, *13*, 3093–3103. <https://doi.org/10.2147/ijn.s162087>.
- (80) Cheng, C.; Peng, S.; Li, Z.; Zou, L.; Liu, W.; Liu, C. Improved bioavailability of curcumin in liposomes prepared using a pH-driven, organic solvent-free, easily scalable process. *RSC Adv.* **2017**, *7*, 25978–25986. <https://doi.org/10.1039/C7RA02861J>.
- (81) Giori, A.; Franceschi, F. Phospholipid complexes of curcumin having improved bioavailability. US 2019 / 0175628 A1, **2019**.
- (82) Zanotto-Filho, A.; Coradini, K.; Braganhol, E.; Schröder, R.; Oliveira, C. M. de; Simões-Pires, A.; Battastini, A. M. O.; Pohlmann, A. R.; Guterres, S. S.; Forcelini, C. M.; *et al.* Curcumin-loaded lipid-core nanocapsules as a strategy to improve pharmacological efficacy of curcumin in glioma treatment. *Eur. J. Pharm. Biopharm.* **2013**, *83*, 156–167. <https://doi.org/10.1016/j.ejpb.2012.10.019>.
- (83) Guri, A.; Gülseren, I.; Corredig, M. Utilization of solid lipid nanoparticles for enhanced delivery of curcumin in cocultures of HT29-MTX and Caco-2 cells. *Food Funct.* **2013**, *4*, 1410–1419. <https://doi.org/10.1039/C3FO60180C>.
- (84) Ganassin, R.; Da Silva, V. C. M.; Araujo, V. H. S.; Tavares, G. R.; Da Silva, P. B.; Cáceres-Vélez, P. R.; Porcel, J. E. M.; Rodrigues, M. C.; Andreozzi, P.; Fernandes, R. P.; *et al.* Solid lipid nanoparticles loaded with curcumin: development and in vitro toxicity against CT26 cells. *Nanomedicine* **2022**, *17*, 167–179. <https://doi.org/10.2217/nnm-2021-0229>.
- (85) Gupta, T.; Singh, J.; Kaur, S.; Sandhu, S.; Singh, G.; Kaur, I. P. Enhancing Bioavailability and Stability of Curcumin Using Solid Lipid Nanoparticles (CLEN): A Covenant for Its Effectiveness. *Front. Bioeng. Biotechnol.* **2020**, *8*, 879–893. <https://doi.org/10.3389/fbioe.2020.00879>.

- (86) Wang, T.; Ma, X.; Lei, Y.; Luo, Y. Solid lipid nanoparticles coated with cross-linked polymeric double layer for oral delivery of curcumin. *Colloids Surf. B: Biointerfaces* **2016**, *148*, 1–11. <https://doi.org/10.1016/j.colsurfb.2016.08.047>.
- (87) Hyun, J. E.; Yi, H.-Y.; Hong, G.-P.; Chun, J.-Y. Digestion stability of curcumin-loaded nanostructured lipid carrier. *LWT* **2022**, *162*, 113474–113483. <https://doi.org/10.1016/j.lwt.2022.113474>.
- (88) Zhang, Y.; Zhao, M.; Ning, Z.; Yu, S.; Tang, N.; Zhou, F. Development of a Sono-Assembled, Bifunctional Soy Peptide Nanoparticle for Cellular Delivery of Hydrophobic Active Cargoes. *J. Agric. Food Chem.* **2018**, *66*, 4208–4218. <https://doi.org/10.1021/acs.jafc.7b05889>.
- (89) Chen, F.-P.; Li, B.-S.; Tang, C.-H. Nanocomplexation between Curcumin and Soy Protein Isolate: Influence on Curcumin Stability/Bioaccessibility and in Vitro Protein Digestibility. *J. Agric. Food Chem.* **2015**, *63*, 3559–3569. <https://doi.org/10.1021/acs.jafc.5b00448>.
- (90) Chen, F.-P.; Li, B.-S.; Tang, C.-H. Nanocomplexation of soy protein isolate with curcumin: Influence of ultrasonic treatment. *Food Res. Int.* **2015**, *75*, 157–165. <https://doi.org/10.1016/j.foodres.2015.06.009>.
- (91) Tapal, A.; Tikku, P. K. Complexation of curcumin with soy protein isolate and its implications on solubility and stability of curcumin. *Food Chem.* **2012**, *130*, 960–965. <https://doi.org/10.1016/j.foodchem.2011.08.025>.
- (92) Wang, Y.; Sun, R.; Xu, X.; Du, M.; Zhu, B.; Wu, C. Structural interplay between curcumin and soy protein to improve the water-solubility and stability of curcumin. *Int. J. Biol. Macromol.* **2021**, *193*, 1471–1480. <https://doi.org/10.1016/j.ijbiomac.2021.10.210>.
- (93) Li, H.; Zhang, X.; Zhao, C.; Zhang, H.; Chi, Y.; Wang, L.; Bai, S. Entrapment of curcumin in soy protein isolate using the pH-driven method: Nanoencapsulation and formation mechanism. *LWT* **2022**, *153*, 112480–112492. <https://doi.org/10.1016/j.lwt.2021.112480>.
- (94) Vijayan, U. K.; Shah, N. N.; Muley, A. B.; Singhal, R. S. Complexation of curcumin using proteins to enhance aqueous solubility and bioaccessibility: Pea protein vis-à-vis whey protein. *J. Food Eng.* **2021**, *292*, 110258–110269. <https://doi.org/10.1016/j.jfoodeng.2020.110258>.
- (95) Ren, J.; Wu, H.; Lu, Z.; Qin, Q.; Jiao, X.; Meng, G.; Liu, W.; Li, G. pH-driven preparation of pea protein isolate-curcumin nanoparticles effectively enhances antitumor activity. *Int. J. Biol. Macromol.* **2024**, *256*, 128383–128400. <https://doi.org/10.1016/j.ijbiomac.2023.128383>.
- (96) Guo, Q.; Bayram, I.; Zhang, W.; Su, J.; Shu, X.; Yuan, F.; Mao, L.; Gao, Y. Fabrication and characterization of curcumin-loaded pea protein isolate-surfactant complexes at neutral pH. *Food Hydrocoll.* **2021**, *111*, 106214–106225. <https://doi.org/10.1016/j.foodhyd.2020.106214>.
- (97) Liu, H.; Wang, Z.; Xu, J.; Ji, F.; Luo, S.; Zhong, X.; Zhao, Y.; Zheng, Z. Self-assembled pea vicilin nanoparticles as nanocarriers for improving the antioxidant activity, environmental stability and sustained-release property of curcumin. *J. Sci. Food Agric.* **2024**, *104*, 2467–2476. <https://doi.org/10.1002/jsfa.13132>.
- (98) Pan, K.; Luo, Y.; Gan, Y.; Baek, S. J.; Zhong, Q. pH-driven encapsulation of curcumin in self-assembled casein nanoparticles for enhanced dispersibility and bioactivity. *Soft Matter* **2014**, *10*, 6820–6830. <https://doi.org/10.1039/C4SM00239C>.

- (99) Zhan, X.; Dai, L.; Zhang, L.; Gao, Y. Entrapment of curcumin in whey protein isolate and zein composite nanoparticles using pH-driven method. *Food Hydrocoll.* **2020**, *106*, 105839–105850. <https://doi.org/10.1016/j.foodhyd.2020.105839>.
- (100) Kevij, T. H.; Salami, M.; Mohammadian, M.; Khodadadi, M. Fabrication and investigation of physicochemical, food simulant release, and antioxidant properties of whey protein isolate-based films activated by loading with curcumin through the pH-driven method. *Food Hydrocoll.* **2020**, *108*, 106026–106035. <https://doi.org/10.1016/j.foodhyd.2020.106026>.
- (101) Wang, Y.; Zhang, L.; Wang, P.; Xu, X.; Zhou, G. pH-shifting encapsulation of curcumin in egg white protein isolate for improved dispersity, antioxidant capacity and thermal stability. *Food Res. Int.* **2020**, *137*, 109366–109378. <https://doi.org/10.1016/j.foodres.2020.109366>.
- (102) Liu, Y.; Cai, Y.; Ying, D.; Fu, Y.; Xiong, Y.; Le, X. Ovalbumin as a carrier to significantly enhance the aqueous solubility and photostability of curcumin: Interaction and binding mechanism study. *Int. J. Biol. Macromol.* **2018**, *116*, 893–900. <https://doi.org/10.1016/j.ijbiomac.2018.05.089>.
- (103) Fu, J.-J.; Sun, C.; Tan, Z.-F.; Zhang, G.-Y.; Chen, G.-B.; Song, L. Nanocomplexes of curcumin and glycated bovine serum albumin: The formation mechanism and effect of glycation on their physicochemical properties. *Food Chem.* **2022**, *368*, 130651–130661. <https://doi.org/10.1016/j.foodchem.2021.130651>.
- (104) Liu, F.; Tang, C.-H. Soy protein nanoparticle aggregates as pickering stabilizers for oil-in-water emulsions. *J. Agric. Food Chem.* **2013**, *61*, 8888–8898. <https://doi.org/10.1021/jf401859y>.
- (105) Chen, M.; Li, R.; Gao, Y.; Zheng, Y.; Liao, L.; Cao, Y.; Li, J.; Zhou, W. Encapsulation of Hydrophobic and Low-Soluble Polyphenols into Nanoliposomes by pH-Driven Method: Naringenin and Naringin as Model Compounds. *Foods* **2021**, *10*, 963–978. <https://doi.org/10.3390/foods10050963>.
- (106) Dai, L.; Zhou, H.; Wei, Y.; Gao, Y.; McClements, D. J. Curcumin encapsulation in zein-rhamnolipid composite nanoparticles using a pH-driven method. *Food Hydrocoll.* **2019**, *93*, 342–350. <https://doi.org/10.1016/j.foodhyd.2019.02.041>.
- (107) Patel, A.; Hu, Y.; Tiwari, J. K.; Velikov, K. P. Synthesis and characterisation of zein–curcumin colloidal particles. *Soft Matter* **2010**, *6*, 6192–6199. <https://doi.org/10.1039/C0SM00800A>.
- (108) Wang, N.; Fan, H.; Wang, J.; Wang, H.; Liu, T. Fabrication and characterization of curcumin-loaded composite nanoparticles based on high-hydrostatic-pressure-treated zein and pectin: Interaction mechanism, stability, and bioaccessibility. *Food Chem.* **2024**, *446*, 138286–138298. <https://doi.org/10.1016/j.foodchem.2023.138286>.
- (109) Yuan, Y.; Huang, J.; He, S.; Ma, M.; Wang, D.; Xu, Y. One-step self-assembly of curcumin-loaded zein/sophorolipid nanoparticles: physicochemical stability, redispersibility, solubility and bioaccessibility. *Food Funct.* **2021**, *12*, 5719–5730. <https://doi.org/10.1039/D1FO00942G>.
- (110) Yuan, Y.; Xiao, J.; Zhang, P.; Ma, M.; Wang, D.; Xu, Y. Development of pH-driven zein/tea saponin composite nanoparticles for encapsulation and oral delivery of curcumin. *Food Chem.* **2021**, *364*, 130401–130410. <https://doi.org/10.1016/j.foodchem.2021.130401>.

- (111) Cheng, Y.; Chen, X.; Lin, Q.; Wang, J.; Chen, Y.; Mo, H.; Chen, M.; Xu, Z.; Wen, L.; Jiao, Y. Improvement effect of different polysaccharides on the functional properties of curcumin-loaded rice protein hydrolysate nanoparticles. *LWT* **2024**, *204*, 116471–116480. <https://doi.org/10.1016/j.lwt.2024.116471>.
- (112) Mehryar, L.; Esmaili, M.; Zeynali, F.; Imani, M.; Sadeghi, R. Fabrication and characterization of sunflower protein isolate nanoparticles, and their potential for encapsulation and sustainable release of curcumin. *Food Chem.* **2021**, *355*, 129572–129585. <https://doi.org/10.1016/j.foodchem.2021.129572>.
- (113) Hadidi, M.; Boostani, S.; Jafari, S. Pea proteins as emerging biopolymers for the emulsification and encapsulation of food bioactives. *Food Hydrocoll.* **2022**, *126*, 107474–107490. <https://doi.org/10.1016/j.foodhyd.2021.107474>.
- (114) Lam, A. C. Y.; Can Karaca, A.; Tyler, R. T.; Nickerson, M. T. Pea protein isolates: Structure, extraction, and functionality. *Food Rev. Int.* **2018**, *34*, 126–147. <https://doi.org/10.1080/87559129.2016.1242135>.
- (115) Lu, Z. X.; He, J. F.; Zhang, Y. C.; Bing, D. J. Composition, physicochemical properties of pea protein and its application in functional foods. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 2593–2605. <https://doi.org/10.1080/10408398.2019.1651248>.
- (116) Tanger, C.; Engel, J.; Kulozik, U. Influence of extraction conditions on the conformational alteration of pea protein extracted from pea flour. *Food Hydrocoll.* **2020**, *107*, 105949–105964. <https://doi.org/10.1016/j.foodhyd.2020.105949>.
- (117) Okagu, O. D.; Udenigwe, C. C. Molecular Interactions of Pea Globulin, Albumin and Glutelin With Curcumin: Formation and Gastric Release Mechanisms of Curcumin-loaded Bio-nanocomplexes. *Food Biophys.* **2022**, *17*, 10–25. <https://doi.org/10.1007/s11483-021-09697-5>.
- (118) Zhang, S. Fabrication of novel biomaterials through molecular self-assembly. *Nat. Biotechnol.* **2003**, *21*, 1171–1178. <https://doi.org/10.1038/nbt874>.
- (119) Ge, J.; Sun, C.-X.; Corke, H.; Gul, K.; Gan, R.-Y.; Fang, Y. The health benefits, functional properties, modifications, and applications of pea (*Pisum sativum* L.) protein: Current status, challenges, and perspectives. *Compr. Rev. Food Sci. Food Saf.* **2020**, *19*, 1835–1876. <https://doi.org/10.1111/1541-4337.12573>.
- (120) Ma, K. K.; Greis, M.; Lu, J.; Nolden, A. A.; McClements, D. J.; Kinchla, A. J. Functional Performance of Plant Proteins. *Foods* **2022**, *11*, 594–617. <https://doi.org/10.3390/foods11040594>.
- (121) Teng, Z.; Luo, Y.; Wang, Q. Nanoparticles synthesized from soy protein: preparation, characterization, and application for nutraceutical encapsulation. *J. Agric. Food Chem.* **2012**, *60*, 2712–2720. <https://doi.org/10.1021/jf205238x>.
- (122) Han, S.; Cui, F.; McClements, D. J.; Xu, X.; Ma, C.; Wang, Y.; Liu, X.; Liu, F. Structural Characterization and Evaluation of Interfacial Properties of Pea Protein Isolate–EGCG Molecular Complexes. *Foods* **2022**, *11*, 2895–2912. <https://doi.org/10.3390/foods11182895>.

- (123) Sinha, A. K.; Verma, S. C.; Sharma, U. K. Development and validation of an RP-HPLC method for quantitative determination of vanillin and related phenolic compounds in *Vanilla planifolia*. *J. Sep. Science* **2007**, *30*, 15–20. <https://doi.org/10.1002/jssc.200600193>.
- (124) Clayton, K. N.; Salameh, J. W.; Wereley, S. T.; Kinzer-Ursem, T. L. Physical characterization of nanoparticle size and surface modification using particle scattering diffusometry. *Biomicrofluidics* **2016**, *10*, 54107–54122. <https://doi.org/10.1063/1.4962992>.
- (125) Minekus, M.; Alving, M.; Alvito, P.; Ballance, S.; Bohn, T.; Bourlieu, C.; Carrière, F.; Boutrou, R.; Corredig, M.; Dupont, D.; *et al.* A standardised static in vitro digestion method suitable for food – an international consensus. *Food Funct.* **2014**, *5*, 1113–1124. <https://doi.org/10.1039/c3fo60702j>.
- (126) *STOE-WinXPOW*; STOE & Cie GmbH: Darmstadt, **2011**.
- (127) Miles, A. J.; Ramalli, S. G.; Wallace, B. A. DichroWeb, a website for calculating protein secondary structure from circular dichroism spectroscopic data. *Protein Sci.* **2022**, *31*, 37–46. <https://doi.org/10.1002/pro.4153>.
- (128) Xu, S.; Dai, W.-G. Drug precipitation inhibitors in supersaturable formulations. *Int. J. Pharm.* **2013**, *453*, 36–43. <https://doi.org/10.1016/j.ijpharm.2013.05.013>.
- (129) Quan, T. H.; Benjakul, S.; Sae-leaw, T.; Balange, A. K.; Maqsood, S. Protein–polyphenol conjugates: Antioxidant property, functionalities and their applications. *Trends Food Sci. Technol.* **2019**, *91*, 507–517. <https://doi.org/10.1016/j.tifs.2019.07.049>.
- (130) Tang, C.-H.; Choi, S.-M.; Ma, C.-Y. Study of thermal properties and heat-induced denaturation and aggregation of soy proteins by modulated differential scanning calorimetry. *Int. J. Biol. Macromol.* **2007**, *40*, 96–104. <https://doi.org/10.1016/j.ijbiomac.2006.06.013>.
- (131) Jiang, J.; Wang, Q.; Xiong, Y. L. A pH shift approach to the improvement of interfacial properties of plant seed proteins. *Curr. Opin. Food Sci.* **2018**, *19*, 50–56. <https://doi.org/10.1016/j.cofs.2018.01.002>.
- (132) Creusot, N.; Wierenga, P. A.; Laus, M. C.; Giuseppin, M. L. F.; Gruppen, H. Rheological properties of patatin gels compared with β -lactoglobulin, ovalbumin, and glycinin. *J. Sci. Food Agric.* **2011**, *91*, 253–261. <https://doi.org/10.1002/jsfa.4178>.
- (133) O’Kane, F. E.; Vereijken, J. M.; Gruppen, H.; van Boekel, M. A. Gelation Behavior of Protein Isolates Extracted from 5 Cultivars of *Pisum sativum* L. *J. Food Sci.* **2005**, *70*, C132–C137. <https://doi.org/10.1111/j.1365-2621.2005.tb07073.x>.
- (134) Sun, X. D.; Arntfield, S. D. Gelation properties of salt-extracted pea protein induced by heat treatment. *Food Res. Int.* **2010**, *43*, 509–515. <https://doi.org/10.1016/j.foodres.2009.09.039>.
- (135) Shand, P. J.; Ya, H.; Pietrasik, Z.; Wanasundara, P. Physicochemical and textural properties of heat-induced pea protein isolate gels. *Food Chem.* **2007**, *102*, 1119–1130. <https://doi.org/10.1016/j.foodchem.2006.06.060>.
- (136) Sun, X. D.; Arntfield, S. D. Dynamic oscillatory rheological measurement and thermal properties of pea protein extracted by salt method: Effect of pH and NaCl. *J. Food Eng.* **2011**, *105*, 577–582. <https://doi.org/10.1016/j.jfoodeng.2011.03.008>.

- (137) Messiou, J.-L.; Sok, N.; Assifaoui, A.; Saurel, R. Thermal Denaturation of Pea Globulins (*Pisum sativum* L.)—Molecular Interactions Leading to Heat-Induced Protein Aggregation. *J. Agric. Food Chem.* **2013**, *61*, 1196–1204. <https://doi.org/10.1021/jf303739n>.
- (138) Peng, W.; Kong, X.; Chen, Y.; Zhang, C.; Yang, Y.; Hua, Y. Effects of heat treatment on the emulsifying properties of pea proteins. *Food Hydrocoll.* **2016**, *52*, 301–310. <https://doi.org/10.1016/j.foodhyd.2015.06.025>.
- (139) Wang, W. Protein aggregation and its inhibition in biopharmaceutics. *Int. J. Pharm.* **2005**, *289*, 1–30. <https://doi.org/10.1016/j.ijpharm.2004.11.014>.
- (140) Chandler, D. Interfaces and the driving force of hydrophobic assembly. *Nature* **2005**, *437*, 640–647. <https://doi.org/10.1038/nature04162>.
- (141) Grossmann, L.; McClements, D. J. Current insights into protein solubility: A review of its importance for alternative proteins. *Food Hydrocoll.* **2023**, *137*, 108416–108437. <https://doi.org/10.1016/j.foodhyd.2022.108416>.
- (142) Zhi, Z.; Yan, L.; Li, H.; Dewettinck, K.; van der Meeren, P.; Liu, R.; van Bockstaele, F. A combined approach for modifying pea protein isolate to greatly improve its solubility and emulsifying stability. *Food Chem.* **2022**, *380*, 131832–131841. <https://doi.org/10.1016/j.foodchem.2021.131832>.
- (143) Pezeshk, S.; Rezaei, M.; Hosseini, H.; Abdollahi, M. Impact of pH-shift processing combined with ultrasonication on structural and functional properties of proteins isolated from rainbow trout by-products. *Food Hydrocoll.* **2021**, *118*, 106768–106785. <https://doi.org/10.1016/j.foodhyd.2021.106768>.
- (144) Rabiei, M.; Palevicius, A.; Dashti, A.; Nasiri, S.; Monshi, A.; Vilkauskas, A.; Janusas, G. Measurement Modulus of Elasticity Related to the Atomic Density of Planes in Unit Cell of Crystal Lattices. *Materials* **2020**, *13*, 4380–4397. <https://doi.org/10.3390/ma13194380>.
- (145) Pawar, Y. B.; Shete, G.; Popat, D.; Bansal, A. K. Phase behavior and oral bioavailability of amorphous Curcumin. *Eur. J. Pharm. Sci.* **2012**, *47*, 56–64. <https://doi.org/10.1016/j.ejps.2012.05.003>.
- (146) Barik, A.; Priyadarsini, K. I.; Mohan, H. Photophysical Studies on Binding of Curcumin to Bovine Serum Albumin. *Photochem. Photobiol.* **2003**, *77*, 597–603. [https://doi.org/10.1562/0031-8655\(2003\)0770597PSOBOC2.0.CO2](https://doi.org/10.1562/0031-8655(2003)0770597PSOBOC2.0.CO2).
- (147) Chignell, C. F.; Bilskj, P.; Reszka, K. J.; Motten, A. G.; Sik, R. H.; Dahl, T. A. Spectral and photochemical properties of curcumin. *Photochem. Photobiol.* **1994**, *59*, 295–302. <https://doi.org/10.1111/j.1751-1097.1994.tb05037.x>.
- (148) Khopde, S. M.; Priyadarsini, K. I.; Palit, D. K.; Mukherjee, T. Effect of Solvent on the Excited-state Photophysical Properties of Curcumin. *Photochem. Photobiol.* **2000**, *72*, 625–631. [https://doi.org/10.1562/0031-8655\(2000\)0720625EOSOTE2.0.CO2](https://doi.org/10.1562/0031-8655(2000)0720625EOSOTE2.0.CO2).
- (149) Nardo, L.; Paderno, R.; Andreoni, A.; Másson, M.; Haukvik, T.; Tønnesen, H. H. Role of H-bond formation in the photoreactivity of curcumin. *J. Spectrosc.* **2008**, *22*, 928407. <https://doi.org/10.3233/SPE-2008-0335>.

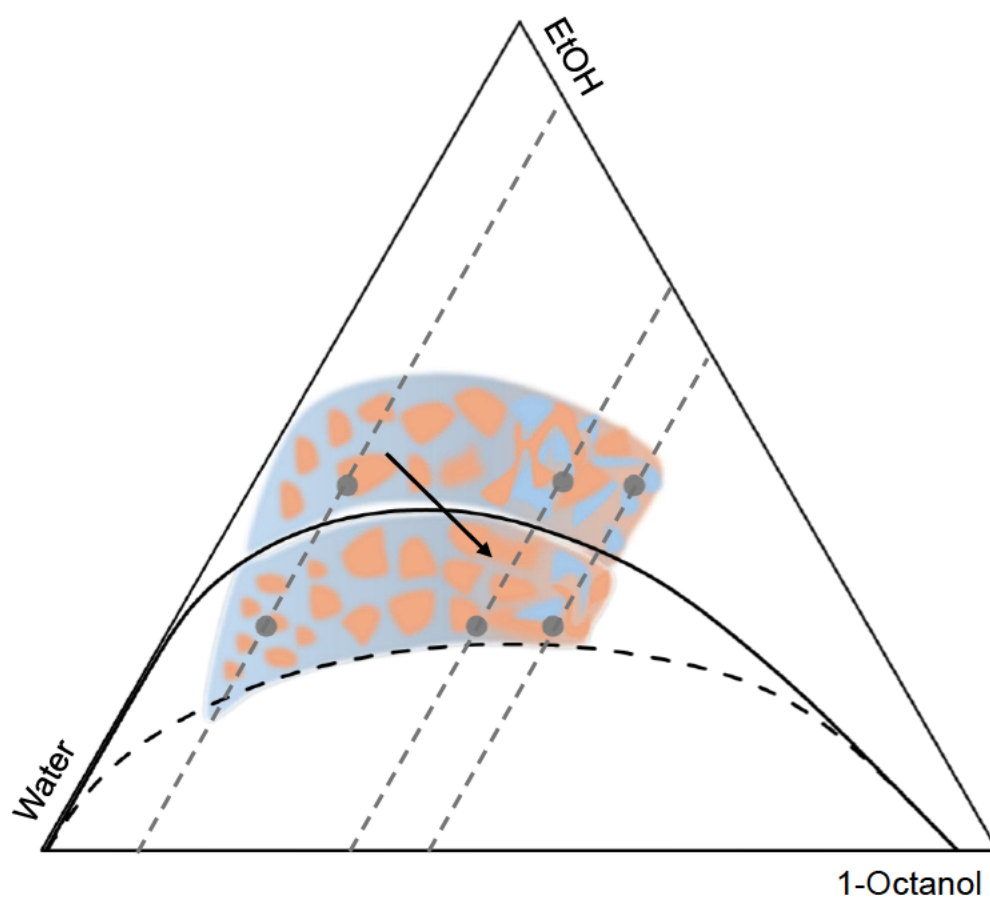
- (150) Patra, D.; Barakat, C. Synchronous fluorescence spectroscopic study of solvatochromic curcumin dye. *Spectrochim. Acta - A: Mol. Biomol. Spectrosc.* **2011**, *79*, 1034–1041. <https://doi.org/10.1016/j.saa.2011.04.016>.
- (151) Kazakova, O.; Lipkovska, N.; Barvinchenko, V. Keto-enol tautomerism of curcumin in the preparation of nanobiocomposites with fumed silica. *Spectrochim. Acta - A: Mol. Biomol. Spectrosc.* **2022**, *277*, 121287–121295. <https://doi.org/10.1016/j.saa.2022.121287>.
- (152) Manolova, Y.; Deneva, V.; Antonov, L.; Drakalska, E.; Momekova, D.; Lambov, N. The effect of the water on the curcumin tautomerism: A quantitative approach. *Spectrochim. Acta - A: Mol. Biomol. Spectrosc.* **2014**, *132*, 815–820. <https://doi.org/10.1016/j.saa.2014.05.096>.
- (153) Bhatia, N. K.; Kishor, S.; Katyal, N.; Gogoi, P.; Narang, P.; Deep, S. Effect of pH and temperature on conformational equilibria and aggregation behaviour of curcumin in aqueous binary mixtures of ethanol. *RSC Adv.* **2016**, *6*, 103275–103288. <https://doi.org/10.1039/C6RA24256A>.
- (154) Adhikary, R.; Carlson, P. J.; Kee, T. W.; Petrich, J. W. Excited-State Intramolecular Hydrogen Atom Transfer of Curcumin in Surfactant Micelles. *J. Phys. Chem. B* **2010**, *114*, 2997–3004. <https://doi.org/10.1021/jp9101527>.
- (155) Dutta, A.; Boruah, B.; Manna, A. K.; Gohain, B.; Saikia, P. M.; Dutta, R. K. Stabilization of diketo tautomer of curcumin by premicellar anionic surfactants: UV–Visible, fluorescence, tensiometric and TD-DFT evidences. *Spectrochim. Acta - A: Mol. Biomol. Spectrosc.* **2013**, *104*, 150–157. <https://doi.org/10.1016/j.saa.2012.11.048>.
- (156) Guo, Q.; Su, J.; Shu, X.; Yuan, F.; Mao, L.; Liu, J.; Gao, Y. Fabrication, structural characterization and functional attributes of polysaccharide-surfactant-protein ternary complexes for delivery of curcumin. *Food Chem.* **2021**, *337*, 128019–128029. <https://doi.org/10.1016/j.foodchem.2020.128019>.
- (157) Hudson, E. A.; Paula, H. M. C. de; Da Silva, R. M.; dos Santos Pires, A. C.; Da Silva, L. H. M. Curcumin-micellar casein multisite interactions elucidated by surface plasmon resonance. *Int. J. Biol. Macromol.* **2019**, *133*, 860–866. <https://doi.org/10.1016/j.ijbiomac.2019.04.166>.
- (158) Sahu, A.; Kasoju, N.; Bora, U. Fluorescence Study of the Curcumin–Casein Micelle Complexation and Its Application as a Drug Nanocarrier to Cancer Cells. *Biomacromolecules* **2008**, *9*, 2905–2912. <https://doi.org/10.1021/bm800683f>.
- (159) Yang, M.; Wu, Y.; Li, J.; Zhou, H.; Wang, X. Binding of Curcumin with Bovine Serum Albumin in the Presence of ι -Carrageenan and Implications on the Stability and Antioxidant Activity of Curcumin. *J. Agric. Food Chem.* **2013**, *61*, 7150–7155. <https://doi.org/10.1021/jf401827x>.
- (160) Sadeghi, R.; Moosavi-Movahedi, A. A.; Emam-jomeh, Z.; Kalbasi, A.; Razavi, S. H.; Karimi, M.; Kokini, J. The effect of different desolvating agents on BSA nanoparticle properties and encapsulation of curcumin. *J. Nanopart. Res.* **2014**, *16*, 2565–2579. <https://doi.org/10.1007/s11051-014-2565-1>.
- (161) Pan, K.; Chen, H.; Baek, S. J.; Zhong, Q. Self-assembled curcumin-soluble soybean polysaccharide nanoparticles: Physicochemical properties and in vitro anti-proliferation activity against cancer cells. *Food Chem.* **2018**, *246*, 82–89. <https://doi.org/10.1016/j.foodchem.2017.11.002>.

- (162) Desobry, S. A.; Netto, F. M.; Labuza, T. P. Comparison of Spray-drying, Drum-drying and Freeze-drying for β -Carotene Encapsulation and Preservation. *J. Food Sci.* **1997**, *62*, 1158–1162. <https://doi.org/10.1111/j.1365-2621.1997.tb12235.x>.
- (163) Dong, H.; Wang, P.; Yang, Z.; Li, R.; Xu, X.; Shen, J. Dual improvement in curcumin encapsulation efficiency and lyophilized complex dispersibility through ultrasound regulation of curcumin–protein assembly. *Ultrason. Sonochem.* **2022**, *90*, 106188–106198. <https://doi.org/10.1016/j.ultsonch.2022.106188>.
- (164) Fong, S. Y. K.; Ibisogly, A.; Bauer-Brandl, A. Solubility enhancement of BCS Class II drug by solid phospholipid dispersions: Spray drying versus freeze-drying. *Int. J. Pharm.* **2015**, *496*, 382–391. <https://doi.org/10.1016/j.ijpharm.2015.10.029>.
- (165) Heffernan, C.; Ukrainczyk, M.; Zeglinski, J.; Hodnett, B. K.; Rasmuson, Å. C. Influence of Structurally Related Impurities on the Crystal Nucleation of Curcumin. *Cryst. Growth Des.* **2018**, *18*, 4715–4723. <https://doi.org/10.1021/acs.cgd.8b00692>.
- (166) Dei Cas, M.; Ghidoni, R. Dietary Curcumin: Correlation between Bioavailability and Health Potential. *Nutrients* **2019**, *11*, 2147–2161. <https://doi.org/10.3390/nu11092147>.
- (167) Chen, J.; Mosquera-Giraldo, L. I.; Ormes, J. D.; Higgins, J. D.; Taylor, L. S. Bile Salts as Crystallization Inhibitors of Supersaturated Solutions of Poorly Water-Soluble Compounds. *Cryst. Growth Des.* **2015**, *15*, 2593–2597. <https://doi.org/10.1021/acs.cgd.5b00392>.
- (168) Dima, C.; Assadpour, E.; Dima, S.; Jafari, S. M. Nutraceutical nanodelivery; an insight into the bioaccessibility/bioavailability of different bioactive compounds loaded within nanocarriers. *Crit. Rev. Food Sci. Nutr.* **2021**, *61*, 3031–3065. <https://doi.org/10.1080/10408398.2020.1792409>.
- (169) Hu, Y.; Lin, Q.; Zhao, H.; Li, X.; Sang, S.; McClements, D. J.; Long, J.; Jin, Z.; Wang, J.; Qiu, C. Bioaccessibility and bioavailability of phytochemicals: Influencing factors, improvements, and evaluations. *Food Hydrocoll.* **2023**, *135*, 108165–108177. <https://doi.org/10.1016/j.foodhyd.2022.108165>.
- (170) Sharma, A.; Arora, K.; Mohapatra, H.; Sindhu, R. K.; Bulzan, M.; Cavalu, S.; Panesar, G.; Elansary, H. O.; El-Sabrou, A. M.; Mahmoud, E. A.; *et al.* Supersaturation-Based Drug Delivery Systems: Strategy for Bioavailability Enhancement of Poorly Water-Soluble Drugs. *Molecules* **2022**, *27*, 2969–2987. <https://doi.org/10.3390/molecules27092969>.
- (171) Brouwers, J.; Brewster, M. E.; Augustijns, P. Supersaturating Drug Delivery Systems: The Answer to Solubility-Limited Oral Bioavailability? *J. Pharm. Sci.* **2009**, *98*, 2549–2572. <https://doi.org/10.1002/jps.21650>.
- (172) Joshi, P.; Sangamwar, A. T. Stabilizing Supersaturated drug-delivery System Through Mechanism of Nucleation and Crystal Growth Inhibition of Drugs. *Ther. Deliv.* **2018**, *9*, 873–885. <https://doi.org/10.4155/tde-2018-0031>.
- (173) Huang, X.; Brazel, C. S. On the importance and mechanisms of burst release in matrix-controlled drug delivery systems. *J. Control. Release.* **2001**, *73*, 121–136. [https://doi.org/10.1016/S0168-3659\(01\)00248-6](https://doi.org/10.1016/S0168-3659(01)00248-6).

References

- (174) Rahman, M. A.; Ali, A.; Rahamathulla, M.; Salam, S.; Hani, U.; Wahab, S.; Warsi, M. H.; Yusuf, M.; Ali, A.; Mittal, V.; *et al.* Fabrication of Sustained Release Curcumin-Loaded Solid Lipid Nanoparticles (Cur-SLNs) as a Potential Drug Delivery System for the Treatment of Lung Cancer: Optimization of Formulation and In Vitro Biological Evaluation. *Polymers* **2023**, *15*, 542–558. <https://doi.org/10.3390/polym15030542>.
- (175) Jithan, A. V., Madhavi, K., Madhavi, M., Prabhakar, K. Preparation and characterization of albumin nanoparticles encapsulating curcumin intended for the treatment of breast cancer. *Int. J. Pharm. Investig.* **2011**, *1*, 119–125. <https://doi.org/10.4103/2230-973x.82432>.
- (176) Ye, Z.; Wang, Y.; Shen, P.; Sagis, L. M.; Landman, J. Effect of gum Arabic coating on release behavior of curcumin-loaded kafirin and zein composite nanoparticles. *Food Hydrocoll.* **2024**, *156*, 110254–110266. <https://doi.org/10.1016/j.foodhyd.2024.110254>.

Chapter 4 The Influence of Surface-active Compounds on Surfactant-free Microemulsions



This chapter is planned for publication (Rohr, L.; Denk, P.; Blahnik, J.; Müller, E.; Sztucki, M.; Kunz, W. The influence of surface-active compounds on surfactant-free microemulsions. *In preparation*). Dr. Patrick Denk helped with the fitting and evaluation of DLS and SAXS data. Daria Nieder provided support in performing phase diagrams.

4.1. Abstract

Surfactant-free microemulsions (SFME) have experienced great interest in recent years as alternative solvent systems in research and industrial applications. Their mesostructure has been studied in detail in the last decade. However, the influence of additives on the mesostructure has barely been investigated. In the present study, the influence of different surface-active compounds at comparably low concentrations on the position of the binodal and the mesostructure of the system EtOH/Oct/Water is studied. By recording phase diagrams at different additive concentrations, a minor impact of nonionic surfactants on the binodal was observed, while adding ionic surfactants and an ionic hydrotrope significantly decreased the binodal. By performing DLS and SAXS measurements in the monophasic region near the binodal, the mesostructure was analyzed in greater detail. While only minor changes in the structuring were observed in the case of adding nonionic surfactants, more pronounced changes were observed in the case of adding ionic compounds. Based on the findings on the mesostructure, the impact of an additive on the binodal is proposed to depend on the presence or absence of charge, their solubility in the SFME solvent compounds, and, in the case of an ionic surfactant, the hydration of the headgroup. Maximum decrease in the binodal was suggested to result from interfacial charging and strong hydration of the headgroup. This was the case for tested anionic surfactants. Additionally, upon adding ionic surfactants at comparably small concentrations, the pre-Ouzo region was found to be extended to the Oct-rich region, and the critical point is proposed to be shifted toward higher % Oct as well. By systematic investigation of different compound characteristics at different concentrations and linking the macroscale impact to mesoscale structuring, a guideline is proposed to estimate the impact of (surface-active) additives based on classical concepts.

4.2. Fundamental Information

4.2.1. Surfactants, Emulsions, And Conventional Mikroemulsions

Surfactants, short for surface-active agents, are organic compounds characterized by a hydrophilic headgroup and a hydrophobic tail. This amphiphilic property leads to several distinct physicochemical characteristics, including the ability to spontaneously assemble into micelles once the concentration exceeds a certain level in the bulk phase, known as the critical micelle concentration (CMC). Surfactants are typically classified by the nature of their polar head group, with main categories including ionic, nonionic, and zwitterionic surfactants; additional subgroups are distinguished by specific functional and structural features within these categories. Thanks to their amphiphilic nature, they can reduce the interfacial tension, which is called surface tension in water-air systems, between two immiscible phases, such as oil and water, by preferentially adsorbing at the interface¹⁻⁵. Depending on the composition of water, oil, and surfactant, various macro- and mesoscale structures can be observed. A schematic overview is shown in a ternary phase diagram in Figure 4.1.

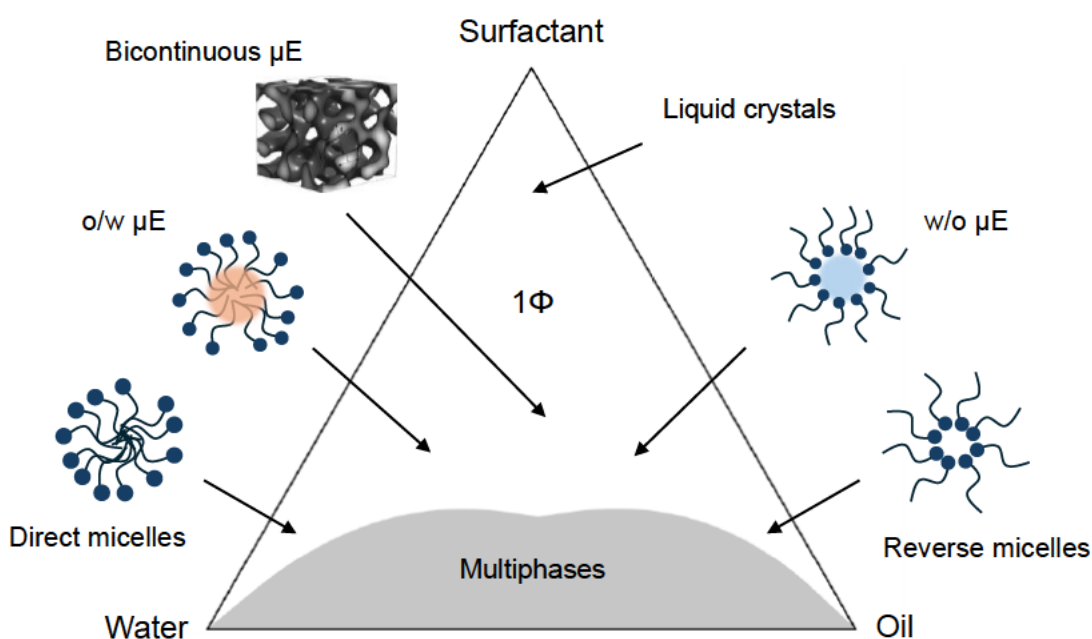


Figure 4.1: Simplified schematic ternary phase diagram of the system surfactant-oil-water with the corresponding mesostructures: direct micelles, oil-in-water microemulsion (o/w μ E), bicontinuous microemulsion (bicontinuous μ E), water-in-oil microemulsion (w/o μ E), and reverse micelles. The 3D view of the bicontinuous μ E was reproduced from Mihailescu et al.⁶ with the permission of AIP Publishing. The representation of the phase diagram is inspired by the work of Sebastian Krickl⁷.

In a system mainly composed of water and oil with a low surfactant concentration, multi-phase systems (grey area) such as emulsions form, with droplet sizes in the micrometer range. The system is only kinetically stabilized by the surfactant, and the droplets tend to coalesce over time, leading to phase separation of the emulsion. In a binary system with a surfactant concentration above the CMC,

either direct or reverse micelles form, depending on the bulk phase, featuring a hydrophobic or hydrophilic core, respectively. As the surfactant content increases and either oil or water is added, the third compound is incorporated into the core, and oil-in-water (o/w) or water-in-oil (w/o) microemulsions (μE) can form. Transitioning from o/w to w/o μE , three-dimensional sponge-like structures, known as bicontinuous μE , appear. At high surfactant concentrations, multiple liquid crystalline phases occur. μEs are thermodynamically stable, isotropic, optically monophasic, and transparent systems, with particle sizes below 100 nm. Only a few surfactants can spontaneously form μEs , and often a cosurfactant is needed, which incorporates into the structure and further reduces surface tension. These systems form well-defined structures with surfactants adsorbed onto the oil-water interface, preventing pseudo-phases from coalescing. However, the structures are not static; they are highly dynamic, constantly disaggregating and reforming due to surfactant exchange between the interface and the outer pseudo-phase. These fluctuations are possible because the surface tension is nearly zero, leaving the bending energy as the main contribution to the free energy of μEs . Since their bending elastic moduli are in the range of thermal energy $k_{\text{B}}T$, these fluctuations are highly temperature-dependent^{1,3–5,8–11}.

The applications of micellar solutions and μE are diverse: surfactants are used as detergents, solubilizers, and emulsifiers in the home care, food, cosmetic, and pharmaceutical industries^{4,12}. Micellar systems and μE are used in enhanced oil recovery⁸, (bio-)catalysis^{13–16}, as template syntheses of polymers and nanoparticles^{17,18}, and as drug delivery systems¹⁹. However, the strong affinity of surfactants for interfaces causes issues such as difficult separation, foaming, and potential toxicity to humans and the environment. Increasing awareness in these areas has driven research toward focusing on less problematic alternatives, such as hydrotropes^{4,20,21}.

4.2.2. Hydrotropes and Surfactant-free Microemulsions

Carl Neuberg first introduced the concept of hydrotropy in 1916^{22,23}. Unlike surfactants, hydrotropes are small amphiphilic molecules that do not form specifically defined aggregates in solution but enhance the solubility of hydrophobic compounds by associating around them, thereby overcompensating the hydrophobic effect. Instead of a CMC, they are sometimes characterized by a minimal hydrotropic concentration (MHC). The MHC indicates the minimum amount of hydrotrope required to make a hydrophobic compound water-soluble. MHCs are typically 1000 times larger than CMCs, indicating the significantly higher solubilization performance of surfactants compared to hydrotropes²⁴. The transition between surfactants and hydrotropes is gradual. Generally, molecules with a longer C-chain than C8 are considered surfactants, while those with shorter chains tend to be classified as hydrotropes. Depending on their structuring behavior in water and the specific system, the term cosurfactant is often used synonymously with hydrotropes²⁵.

Replacing the surfactant in μE with a hydrotrope leads to the formation of surfactant-free microemulsions. Surfactant-free or ultra-flexible microemulsions (SFME or UFME, respectively) are macroscopically homogeneous, transparent, and thermodynamically stable systems with low viscosity consisting of oil, water, and a hydrotrope^{26–29}. Their origins can be traced back to what is now known as the Ouzo effect. It describes the spontaneous emulsification of the liqueur Ouzo, or more scientifically, a mixture of anethol as an oil and EtOH as a hydrotrope, upon the addition of water^{30–32}. The ternary mixture of anethol, EtOH, and water, still in the single-phase region before forming a stable emulsion, is known as SFME or, in the context of its discovery, the “pre-Ouzo” effect^{26,33}. The relation between the Ouzo emulsion and the pre-Ouzo microemulsion has been described by Prévost *et al.*³³ Blahnik *et al.* summarized various SFME systems discussed in the literature, with EtOH/1-Octanol (Oct)/Water being the most extensively studied³⁴. Its structure has been examined in detail over the past decade using static and dynamic light scattering (SLS and DLS, respectively), small- and wide-angle X-ray scattering (SWAXS), and small-angle neutron scattering (SANS)^{27,35}. Polydisperse Oct-rich domains, approximately 2 nm in size, were identified within the pre-Ouzo region on the water-rich side of the phase diagram near the phase boundary^{26,36}. Increasing the Oct proportion while decreasing the water proportion causes the mesostructure to gradually transition into a bicontinuous phase and then into a reverse aggregate phase, similar to classical μEs ^{28,37,38}. These findings have also been supported by scattering data predicted from molecular dynamics (MD) simulations. The simulations delivered a model image describing the structuring in the single-phase region of the system EtOH/Oct/Water, depending on the composition of the single compounds^{28,38,39}. The ternary phase diagram, along with the corresponding snapshots from MD simulations, is shown in Figure 4.2.

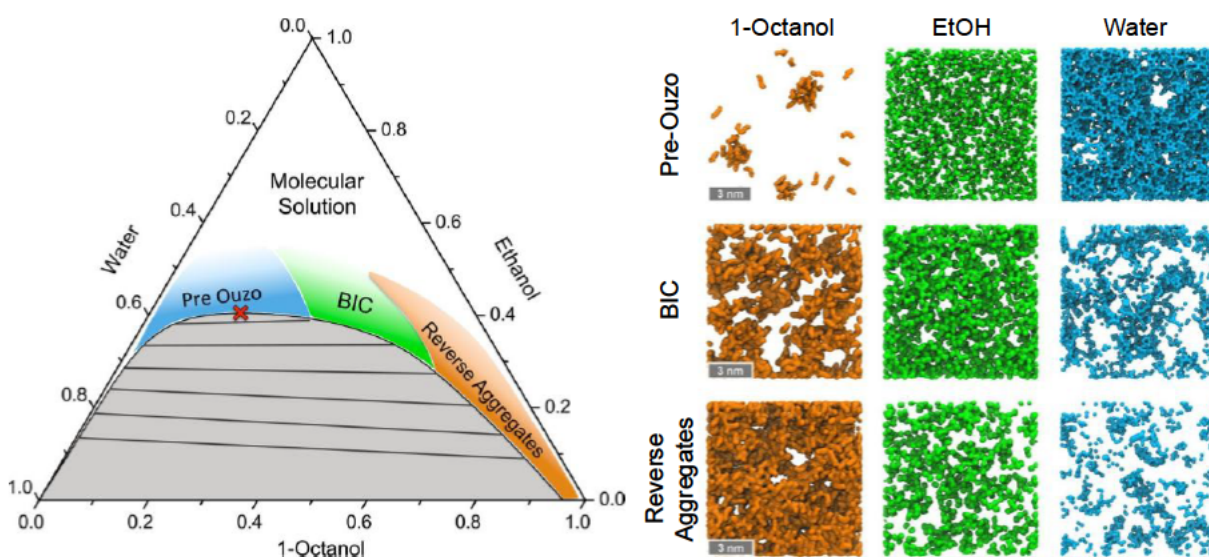


Figure 4.2: Ternary phase diagram of EtOH/Oct/Water with snapshots of the different mesostructures pre-Ouzo, bicontinuous phase (BIC), and reverse aggregates in the regions close to the phase transition border. The black lines in the phase diagram are tie lines in the biphasic region (grey). The red cross marks the critical point. The figure is adapted from Lopian *et al.*²⁸ with modified labelling and reproduced with the permission of ACS.

The investigations described the SFME as a mesoscale solubilization of the hydrotrope-oil pseudophase within a water-hydrotrope pseudophase, with the hydrotrope slightly accumulating at the interface and partitioning between the two domains. Since there are no surfactants, the interface is very weakly stabilized and highly dynamic. As a result, aggregates are less well defined than expected for classical micelles^{28,36,38–41}. This circumstance is one reason why the presence of such mesoscopically structured mixtures was not taken seriously for a long time, despite hints dating back to the 1970s^{42–45}.

However, how is this system stabilized? And can its formation be predicted? Over the past decade, an increasing number of papers have been published aiming to answer these questions using theoretical approaches^{27,46–53}. Zemb *et al.* proposed a model analogous to the Derjaguin–Landau–Verwey–Overbeek (DLVO) approach to describe the free energy ΔG of SFME systems. In classical μE , the DLVO approach describes the colloidal stability of charged surfactant micelles in liquid media, considering the balance between van der Waals attraction and electrostatic repulsion, supplemented by entropic terms related to the flexibility of interface bilayers and the area per surfactant molecule. Since there are no surfactants, there is no electrostatic repulsion or contribution from the area per surfactant molecule to ΔG . Based on MD simulation results, electrostatic repulsion was replaced with short-range repulsion caused by water ordering (free energy of hydration) near the interface and a low hydrotrope concentration gradient (here, EtOH). The area per surfactant molecule was replaced with the mixing entropy, driving the system toward a higher degree of mixing between the phases and thus, smaller domains. The van der Waals contribution was found to be very small. Balancing the competing contributions of hydration and mixing entropy, a shallow free energy minimum (about $0.2 k_B T$ per EtOH molecule) emerges at a correlation length of about 2 nm, aligning with experimental data. This shallow free energy minimum allows the formation of fluctuating, polydisperse microdomains^{27,54}. The contributions to ΔG are schematically depicted in Figure 4.3.

Seo *et al.* applied the Kirkwood-Buff theory to quantify microheterogeneities in binary and ternary systems, and to predict SFME formation in various short-chain alcohols, water, and n-hexane. They found that interactions between water-hexane and alcohol-hexane are not crucial for SFME formation, but water-water, water-alcohol, and alcohol-alcohol interactions are important. Alcohols that promote water aggregation therefore facilitate SFME formation, whereas homogeneous co-solvation of alcohol and water hinders it⁵¹.

The paper by Han *et al.* demonstrates how three complementary models collectively provide a coherent understanding of SFME: at the thermodynamic level, Flory-Huggins analysis provides insights into the ratio of hydrotrope-oil and hydrotrope-water interactions necessary for SFME formation, showing that no SFME can form if the interactions between hydrotrope and oil become too strong. Mesoscopic DPD simulations describe structure formation as a result of the balance between

conservative, dissipative, and random forces. When this balance is disturbed, the system's free energy increases, making phase separation more likely. At the molecular (DFT) level, calculations reveal that the accumulation and directional orientation of EtOH at the interface, together with its polarization within EtOH-Water clusters, induce a net surface charge on the droplets, which suppresses their fusion⁴⁹. Chen *et al.* and Ma *et al.* recently also applied the Flory-Huggins theory to describe the free energy ΔG of SFMEs and highlighted the delicate balance between enthalpy and entropy in forming SFMEs (see Figure 4.3)^{52,53}. Chen *et al.* observed an increase in zeta potential toward more negative values as the system approaches the phase boundary⁵², which was also supported by DFT calculations from Han *et al.*⁴⁹. Theoretical analysis by Chen *et al.* demonstrated that the balance between internal energy and mixing entropy can be affected by changing the components' volume fractions and their interaction parameters⁵². Ma *et al.* expanded on these findings with a fully thermodynamic, extended Flory-Huggins framework. They introduced a new parameter that considers the interaction between the two pseudo-phases and predicts SFME regions in the ternary phase diagram and stable droplet sizes⁵³. These theoretical approaches have enhanced understanding in recent years and have greatly improved the predictability of SFME systems. However, a generalized prediction is still not possible due to the ongoing lack of contributions from individual molecular effects.

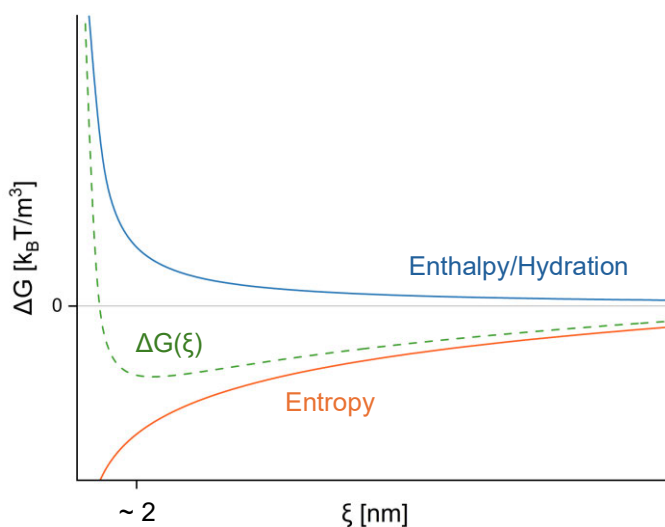


Figure 4.3: The contributions of enthalpy/hydration and entropy to the free energy ΔG of the system, and the resulting shallow minimum at a correlation length (ξ) of approximately 2 nm. The van-der-Waals attraction is neglected. The representation is inspired by Zemb *et al.*²⁷ and Ma *et al.*⁵³.

SFME aggregates were found to be highly sensitive to the addition of electrolytes. Marcus *et al.* studied the impact of various salts on the structure of EtOH/Oct/Water using DLS, SAXS, and SANS. They observed a salting-out effect of EtOH from the aqueous pseudo-phase, driving it toward the interface or into the Oct-phase, leading to overall larger SFME aggregates⁵⁵. In the case of antagonistic salts, meaning salts containing a hydrophilic and a hydrophobic ion^{56–58}, the charge separation at the interface of SFME aggregates stabilizes them electrostatically. This process gradually transforms the aggregates into micelle-like structures with the salt and EtOH serving as surfactant. When low

concentrations of SDS are added to the SFME, EtOH remains the main component at the interface. With increasing SDS concentration, classical mixed micelles form with Oct acting as a cosurfactant⁵⁵. Schöttl and Horinek simulated the effects of 1 mol/L sodium iodide and lithium chloride on EtOH/Oct/Water. They also observed salting-out of EtOH from the aqueous pseudo-phase into the non-polar phase and the interface, along with minimal charging of the interface due to differential ion adsorption. Both effects contributed to the stabilization and formation of larger SFME aggregates⁵⁹. Gao *et al.* very recently studied the influence of ionic strength on the stability of SFME aggregates in EtOH/trans-Anethole/Water via DLS, NTA, and zeta potential measurements. They found that at low ionic strength, the surface charge of SFME nanodomains was most negative, and it vanished at higher ionic strength, resulting in droplet coalescence and phase separation⁶⁰. Marcus *et al.* investigated various salts over a wide range of concentrations and described changes as the system transitions from pure SFME to high-salt conditions, with little focus on low additive concentrations. They also noted a shift in the binodal position when adding SDS, although this was not discussed in detail⁵⁵. The current work builds on that of Marcus *et al.*, focusing on how different surfactants at very low concentrations influence the phase behavior of EtOH/Oct/Water and the mesoscale processes underlying these changes.

SFMEs are not only of academic interest but also have many practical uses. The earliest documented application was as a structured reaction medium for membrane enzymatic reactions^{61–64}. However, so far, there is no evidence that mesostructuring of SFMEs outperforms the solubilization effect of the solvent composition^{65,66}. Schmauser *et al.* recently published a comprehensive review on current developments in the application of SFME systems⁶⁷. SFMEs present an alternative solvent for the solubilization and extraction of natural compounds. Degot *et al.* extracted curcumin from *Curcuma Longa L.* using the SFME EtOH/Triacetin/Water. These constituents are edible and food-approved, making them a more sustainable alternative to harmful solvents and energy-intensive extraction methods^{68,69}. Zhang *et al.* solubilized the vitamin riboflavin and curcumin in the SFME EtOH/1-Octen-3-ol/Water and significantly increased their solubilities⁷⁰. SFMEs have also been used in aqueous insect repellents or observed in fragrance formulations^{71,72}. The mesostructure of SFME has also been reported in the literature to provide nanoreactors for nanoparticle synthesis^{73–76} or polymerization reactions^{77,78}. SFMEs offer an alternative to surfactant-based microemulsions, especially when only subtle compartmentation is required. Unlike surfactants, hydrotropes are weakly surface-active, often easy to separate, inexpensive, widely available, and less likely to cause ecotoxic effects. While surfactant-based systems remain essential for applications requiring well-defined, stable interfaces, SFMEs provide a more flexible and sustainable option, making them attractive for a wide range of future applications³⁴.

4.3. Experimental Section

4.3.1. Materials

Ethanol (EtOH, p.A.), 1-octanol (Oct, $\geq 99\%$), sodium dodecyl sulfate (SDS, electrophoresis grade), lauryltrimethyl ammonium bromide (LTAB, $\geq 98\%$), sodium laurate (NaL, 99-100%), and sodium salicylate (NaSal, $> 99.5\%$) were purchased from Sigma-Aldrich (Darmstadt, Germany). Polyoxyl-10 lauryl ether (C12E10, technical grade) was obtained from Merck (Darmstadt, Germany). Sodium laureth-6 carboxylate (SLEC, trade name Akypo Soft 45 HP, technical grade) was gifted by Kao Chemicals (Emmerich am Rhein, Germany). Glycerol monolaurin (GML, $\approx 90\%$) was provided by the manufacturer proviron (Antwerp, Belgium). All chemicals were used without purification. SLEC was dried to a water content of $< 0.1\%$ (w/w) (measured via coulometric Karl-Fischer titration). Millipore water was obtained from an in-house MilliQ water purification system at a resistivity of 18 M Ω .

4.3.2. Methods

4.3.2.1. (Pseudo-) Ternary Phase Diagrams

(Pseudo-)Ternary phase diagrams were recorded following the procedure established by Clause *et al.*⁷⁹. Binary mixtures of water and hydrotrope or hydrotrope and oil were prepared in % (w/w). The masses were weighed in borosilicate glass screw tubes. After homogenization, the third compound was added dropwise until visible changes in the phase behavior were observed. The surfactants and NaSal were added in % (w/w), calculated as part of EtOH ($\%(additive) = \frac{m(additive)}{m(additive)+m(EtOH)} \cdot 100$).

(Pseudo-)Ternary phase diagrams of EtOH/Oct/Water were recorded at $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$.

4.3.2.2. Dynamic Light Scattering

DLS experiments were performed as described in chapter 2.3.3.1. Samples were filtered using 0.22 μm PTFE membrane filters. Data were fitted using second-order cumulant analysis^{80–82}. The field autocorrelation function is described by equation 4.1. Following the Siegert relation (see equation 1.9), including an adaptation from Frisken *et al.*⁸³, the autocorrelation function is given by equation 4.2. Fitting was performed using an OriginLab implementation written by Patrick Denk.

$$g^1(\tau) = \exp\left(-\Gamma\tau + \left(\frac{\mu_2}{2!}\right) \cdot \tau^2\right) \quad (4.1)$$

$$g^2(\tau) - 1 = \beta \cdot \exp(-2\Gamma\tau) \cdot \left(1 + \left(\frac{\mu_2}{2!}\right) \cdot \tau^2\right)^2 + C \quad (4.2)$$

with μ_2 as the expansion coefficient, β as the apparent coherence factor, and C as a correction parameter for baseline drifts.

4.3.2.3. Small-Angle X-Ray Scattering

Small-angle X-ray Scattering (SAXS) data were collected at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) on the ID02⁸⁴, TRUSAXS, beamline. Beam time was granted under proposal number SC-5453 (DOI: 10.15151/ESRF-ES-1438785010). Samples were measured in a flow-through quartz capillary from Hilgenberg GmbH (Malsfeld, Germany) with an inner diameter of 2 mm and a quartz thickness of 50 µm. The capillary was maintained at 25 °C using a Peltier stage LFI-3751 from Wavelength Electronics (Bozeman, Montana, USA). 2D SAXS patterns were recorded at a sample-to-detector distance of 1 m (q -range = 0.076-7.6 nm⁻¹), at an X-ray energy of 12.23 keV/ λ = 0.101 nm, and a fixed wavelength of 0.099 nm. The detector used was a Dectris (Baden-Daettwil, Switzerland) Eiger2 4M pixel array detector, which simultaneously measured the sample transmission. The raw data were processed as follows: 20 scans were performed per sample, each with an exposure time of 0.1 s. The data were radially averaged, and an empty capillary was used as a background, which was subtracted from the data files. Data were corrected for spatial distortion, flat field, transmitted flux, and electronic and dark noise. Normalization of 1D data was performed using the water level as a standard ($d\sigma/d\Omega$ = 0.0163 cm⁻¹) and dividing by the corresponding path length of 2 mm.

4.3.2.4. Strategy of SAXS Data Evaluation

Fitting was performed using implementations into OriginLab written by Patrick Denk.

Fitting to an Ornstein-Zernike+Lorentzian Broad Peak Model (OZ+BP): Scattering data were fitted using a model introduced by Prévost *et al.*, consisting of an Ornstein-Zernike (OZ) contribution and a broad peak (BP) contribution (equations 4.3 and 4.4). Both contributions are Lorentzian functions, with OZ having its mode at $q=0$ and BP having its mode at intermediate q ^{11,37,85}. The OZ model is a purely statistical model that generally describes scattering due to concentration fluctuations. In the case of SFMEs, these fluctuations are caused by critical fluctuations or polydispersity of aggregates^{37,86}. The BP model describes the correlation signal of one phase in another, emerging as a structure factor peak^{11,87}.

$$I(q) = I_{OZ}(q) + I_{BP}(q) + background \quad (4.3)$$

$$I(q) = \frac{I_{OZ}(0)}{1 + (\xi_{OZ} \cdot q)^2} + \frac{I_{BP}}{[1 + (\xi_{BP} \cdot (q - q_p))^m]^n} + background \quad (4.4)$$

with $I(q)$ as the total scattering intensity in cm⁻¹, $I_{OZ}(0)$ as the scattering intensity at $q=0$, ξ_{OZ} as the OZ correlation length in nm, I_{BP} as the intensity of the peak, ξ_{BP} as the BP correlation length in nm, and q_p as the peak position. The exponents m and n are a generalisation to any power law and are fixed here to $m = 2$ and $n = 1$ to describe a Lorentzian peak with a decay of q^{-2} at high q . The flat scattering background is given in cm⁻¹. For $q_p \neq 0$, both ξ_{OZ} and ξ_{BP} describe correlation lengths, but have different physical meanings. For $q_p = 0$, the BP contribution is identical to the OZ contribution and $\xi_{BP} = \xi_{OZ}$.

With the peak position q_p obtained from the BP contribution, the interaggregate distance or mesh size d can be calculated.

$$d = \frac{2\pi}{q_p} \quad (4.5)$$

Fitting to a modified Teuber-Strey Model (TS): The Teubner-Strey model⁸⁸ is used for two-domain systems and is frequently employed to describe the scattering data of classical microemulsions^{11,89}. The total scattering intensity $I(q)$ is described by the following equation 4.6:

$$I(q) = 10^7 \cdot \text{scale} \cdot \frac{8 \cdot \pi \cdot \langle \eta^2 \rangle \cdot \frac{c_2}{\xi}}{a_2 + c_1 \cdot q^2 + c_2 \cdot q^4} + \text{background} \quad (4.6)$$

with 10^7 as a conversion factor from nm^{-1} to cm^{-1} , scale as a scaling factor, typically set to 1, $\langle \eta^2 \rangle$ as the mean square of the scattering length density fluctuation, ξ as the correlation length, and a_2 , c_1 , and c_2 as parameters depending on ξ and d .

$$a_2 = \left[1 + \left(\frac{2\pi\xi}{d} \right)^2 \right]^2 \quad (4.7)$$

$$c_1 = -2 \cdot \xi^2 \cdot \left(\frac{2\pi\xi}{d} \right)^2 + 2 \cdot \xi^2 \quad (4.8)$$

$$c_2 = \xi^4 \quad (4.9)$$

$\langle \eta^2 \rangle$ is given by equation 4.10 in the case of a two-(pseudo-)phase system of (pseudo-)phases A and B separated by a sharp interface.

$$\langle \eta^2 \rangle = \phi_A \cdot (1 - \phi_A) \cdot (SLD_A - SLD_B)^2 \quad (4.10)$$

with ϕ_A as the volume fraction of (pseudo-)phase A, $SLD_{A/B}$ as the scattering length densities of (pseudo-)phases A and B, and $(SLD_A - SLD_B)^2$ given as the contrast between both (pseudo-)phases.

The model was adapted to the case of pseudo-phase A, consisting of EtOH and water, and pseudo-phase B, consisting of EtOH and Oct. Parameters ϕ_A , SLD_A , and SLD_B are then given by:

$$\phi_A = \phi_W + \phi_E \cdot x_A(E) \quad (4.11)$$

$$SLD_A = \frac{1}{\phi_W + \phi_E \cdot x_A(E)} \cdot (\phi_W \cdot SLD_W + \phi_E \cdot x_A(E) \cdot SLD_E) \quad (4.12)$$

$$SLD_B = \frac{1}{1 - (\phi_W + \phi_E \cdot x_A(E))} \cdot (\phi_O \cdot SLD_O + \phi_E \cdot (1 - x_A(E)) \cdot SLD_E) \quad (4.13)$$

with $\phi_{W/E/O}$ as the volume fractions of water, EtOH, and Oct, $x_A(E)$ as the proportion of EtOH in pseudo-phase A, and $SLD_{W/E/O}$ as the scattering length densities of water, EtOH, and Oct.

4.4. Results and Discussion

The work presented here builds on research initiated by Jonas Blahnik. He investigated how selected electrolytes and surfactants at low concentrations influence the phase behavior of SFMEs within polymerization systems⁹⁰. He continued the research of Marcus *et al.*⁵⁵, who studied the effects of the same components on SFMEs, but at higher concentrations and at specific points in the pre-Ouzo region (without analyzing the phase behavior). In contrast, Blahnik examined the “former” biphasic region of SFMEs. He observed a significant reduction in the binodal and a change in its shape after adding small amounts of the ionic surfactant SDS. DLS measurements near the binodals indicated a shift of the maximum structuring toward the Oct-rich corner, along with increased correlation coefficient and decay time τ upon the addition of SDS. For the nonionic surfactant C12E10, no reduction of the binodal was observed in a preliminary experiment. Blahnik also studied how sodium alkyl sulfates with varying chain lengths and a range of inorganic anions, with sodium as the counterion, affect the phase behavior of the SFME tert-Butanol/Methyl methacrylate/Water. He noted a gradual reduction in the biphasic region with increasing chain length of sodium alkyl sulfates. Adding inorganic salts either had no impact or caused the biphasic region to expand. Summarizing his findings, he concluded that the reduction of the biphasic region results from the combined surface-active and ionic properties of these compounds. Neither surface activity nor ionic character alone is sufficient. He proposed the hypothesis that SDS molecules might incorporate into the fluctuating interface at low concentrations and stabilize the pre-Ouzo aggregates by acting like a co-hydrotrope or an antagonistic salt⁹⁰. The present work extends these studies by systematically analyzing a range of ionic and nonionic surfactants. The experiments include phase diagrams, DLS, and SAXS data to provide deeper insights into mesoscale processes.

4.4.1. The Impact of Surface-active Compounds on the Macroscopic Phase Behavior of EtOH/Oct/Water

The phase behavior of the system EtOH/Oct/Water with various ionic and nonionic surfactants, along with sodium salicylate (NaSal) as an ionic hydrotrope, was studied. The molecular structures of the tested compounds are shown in Figure 4.4. The main criterion for selecting surfactants was comparing ionic and nonionic properties. Therefore, the C12 chain length was kept constant, while variations in the headgroups were introduced. To minimize specific salt effects, Na^+ was fixed as the counterion for anionic surfactants. SLEC includes a relatively large nonionic proportion compared to the carboxylate group and is generally recognized to act as a border compound between nonionic and anionic surfactants^{91–93}. NaSal, as a traditional ionic hydrotrope, combines ionic character and surface activity without the self-aggregation behavior typical of surfactants.

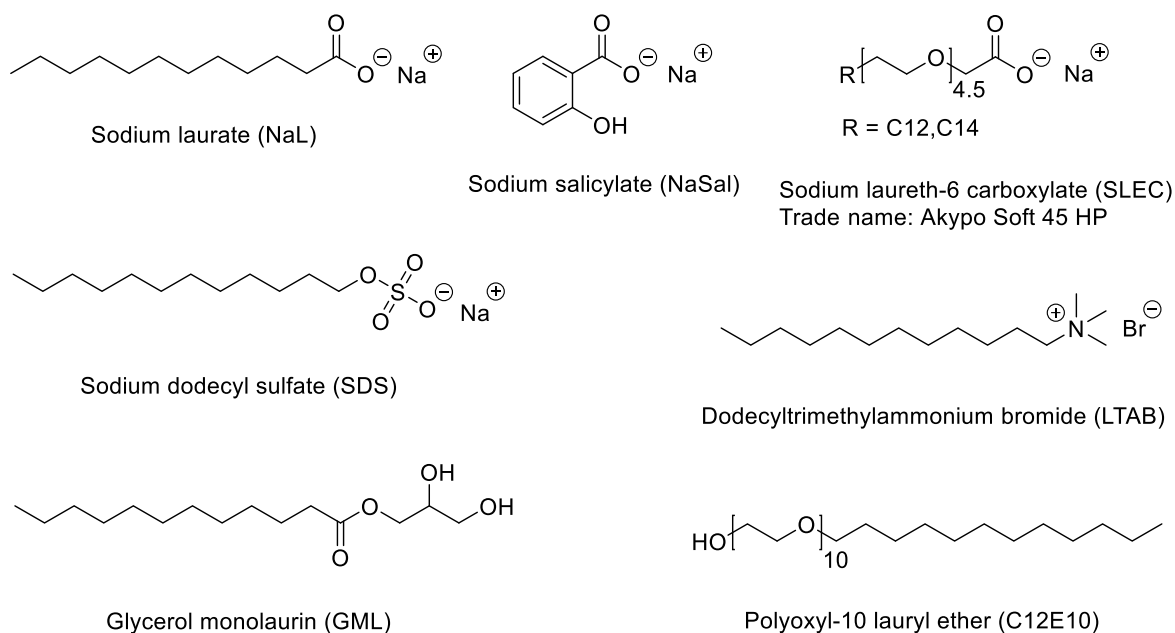


Figure 4.4: Molecular structures of the investigated compounds sodium laurate (NaL), sodium salicylate (NaSal), sodium laureth-6 carboxylate (SLEC), sodium dodecyl sulfate (SDS), dodecyltrimethylammonium bromide (LTAB), glycerol monolaurin (GML), and polyoxyl-10 lauryl ether (C12E10).

The compounds were added in proportion to EtOH in % (w/w). The absolute concentrations of the compounds in the formulations are summarized in Table 4.1. The (pseudo-)ternary phase diagrams of EtOH (+compounds)/Oct/Water are illustrated in Figure 4.5. The position of the critical point (CP) of the EtOH/Oct/Water system is adapted from Jonas Blahnik⁹⁰. The phase diagram of EtOH(+SDS)/Oct/Water is included for comparison.

Adding small amounts of ionic surfactants sodium laurate (NaL, anionic), sodium laureth-6 carboxylate (SLEC, anionic), and dodecyltrimethylammonium bromide (LTAB, cationic) to EtOH gradually reduces the biphasic liquid-liquid region as the surfactant concentration increases. Notably, the reduction of the biphasic region is more significant on the Oct-poor side compared to the Oct-rich side, leading to a more symmetrical shape of the binodal. In the case of SLEC, the biphasic region slightly expands in the Oct-rich corner. NaL proves more effective than SLEC and LTAB in reducing the biphasic region. The addition of glycerol monolaurin (GML, nonionic) does not affect the size or shape of the two-phase region. Adding polyoxyl-10 lauryl ether (C12E10, nonionic) only slightly reduces the two-phase region in the Oct-poor corner, regardless of surfactant concentration. Adding the ionic hydrotrope NaSal reduces the biphasic region uniformly across the binodal, resulting in a shape similar to that of the pure SFME. The results with NaL align with those obtained by Jonas Blahnik using SDS. The experiments on nonionic surfactants GML and C12E10 confirm Blahnik's assumption that nonionic surfactants do not affect the binodal⁹⁰. The experiments with SLEC confirm the preliminary findings with sodium lauryl ether sulphate (SLES) conducted by Blahnik⁹⁰. SLEC, as well as SLES, acts as a mild alternative to NaL and SDS due to its large nonionic moiety and comparably small ionic headgroup.

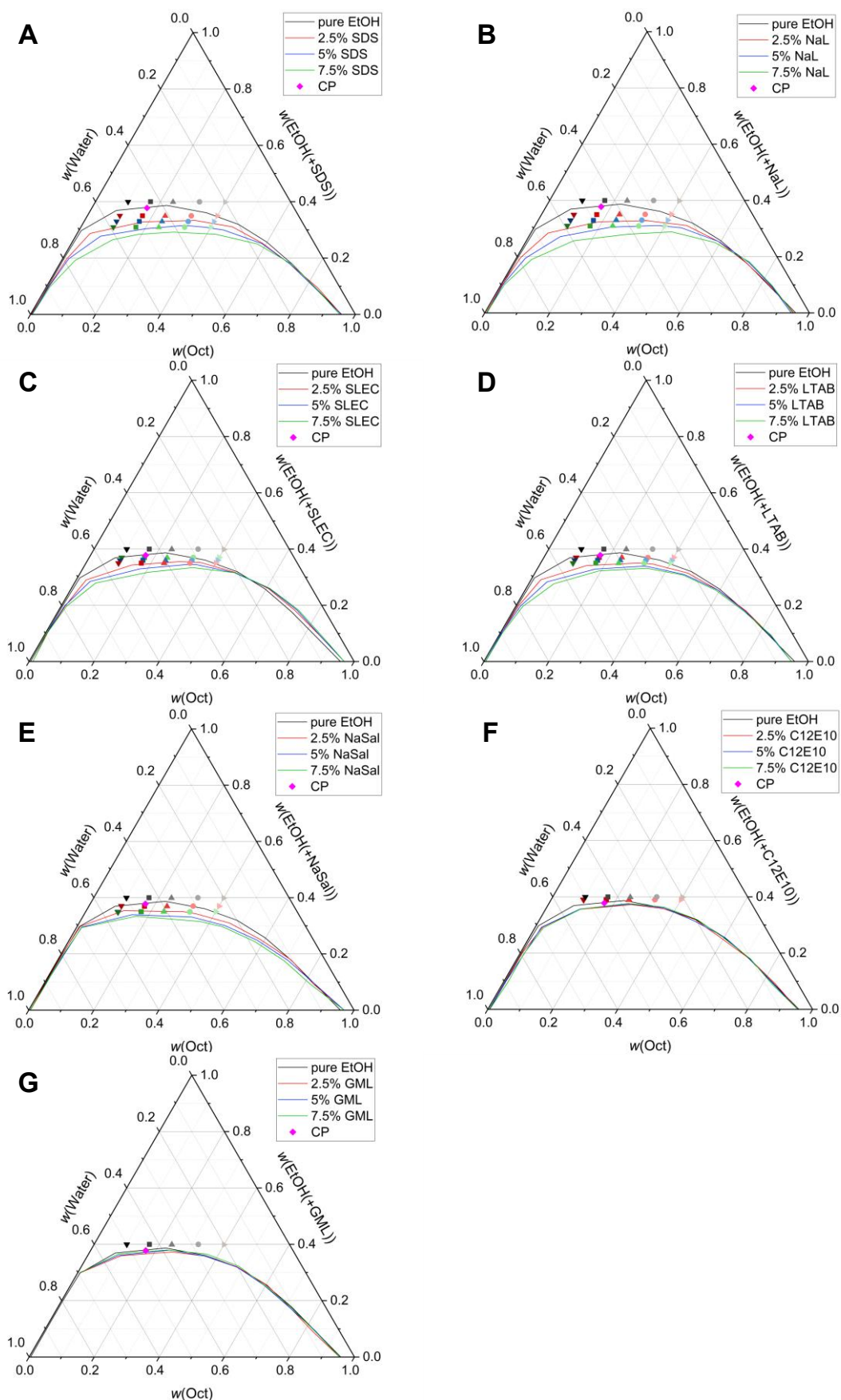


Figure 4.5: (Pseudo-)Ternary phase diagrams of the system EtOH (+compound)/Oct/Water with 2.5%, 5%, and 7.5% NaL (B), SLEC (C), LTAB (D), NaSal (E), C12E10 (F), and GML (G). The pseudo-ternary phase diagram of EtOH(+SDS)/Oct/Water (A) is adapted and reprinted with permission from Jonas Blahnik⁹⁰ for comparison. Symbols indicate measuring points across the binodal. By keeping the EtOH amount constant across the binodal, the surfactant concentration in these samples remains constant. CP was only determined in the pure SFME.

However, measuring concentration by weight percent results in a notably lower $c(\text{SLEC})$ in mol/L due to its higher molecular weight. $c(\text{SLEC})$ at 7.5% (w/w) is equivalent to 5% (w/w) SDS (see Table 4.1). Therefore, the less pronounced impact of SLEC on the water-rich side is probably not only due to its ambivalent molecular structure but also due to its lower concentration in the samples. The impact of NaSal on the phase boundary further supports Blahník's hypothesis that the ionic character is crucial in this phenomenon⁹⁰.

Another property to keep in mind is ion hydration: LTAB has an ammonium headgroup, which, from a Hofmeister perspective, is considered rather soft and weakly hydrated. The carboxylate headgroup present in NaL and SLEC is considered hard and strongly hydrated. The sulfate headgroup of SDS is considered rather soft^{94–96}. This was considered by Lopian *et al.* when adding high concentrations of sulfuric acid to EtOH/Oct/Water. They observed behavior similar to when SLEC is added. They concluded that sulfuric acid stabilizes pre-Ouzo aggregates and destabilizes reverse aggregates by increasing effective repulsion at the pseudo-phase boundaries. This causes salting-in of Oct on the Oct-poor side and a salting-out of water from Oct on the Oct-rich side; the tie lines also shift, indicating salting-in of EtOH into the aqueous pseudophase. The authors discussed two mechanisms: a secondary hydration force arising from the hydration of the added electrolyte, and increased accumulation of EtOH at the interface. Both mechanisms explain the changes in the binodal because they add a free-energy penalty for removing or overlapping the interfacial layers. This prevents coalescence of aggregates into macroscopic phase separation (thus stabilising small pre-Ouzo aggregates) and reduces the ability of the Oct phase to host reverse aggregates (thus destabilising them). SWAXS measurements confirmed the presence of the mesostructure across the binodal in the presence of 2 M sulfuric acid, but were not studied in closer detail²⁸.

Table 4.1: Concentrations of added compounds and CMCs of the surfactants in water at 25 °C. The column headings indicate the proportion of the compound in EtOH by % (w/w). The values in the columns represent the absolute concentrations in the samples in mmol/L. By keeping the EtOH amount constant across the binodal, the surfactant concentration in these samples remains constant. The SDS concentrations are given for comparison.

	c(compound) in proportion of EtOH			CMC in water [mmol/L]
	2.5% (w/w)	5% (w/w)	7.5% (w/w)	
c(SDS) [mmol/L]	26.8 ± 0.5	51.2 ± 1.0	73.1 ± 1.3	7.9-8.0 ⁹⁷
c(NaL) [mmol/L]	34.2 ± 1.1	66.0 ± 1.1	94.6 ± 2.0	23.0-24.4 ⁹⁸
c(LTAB) [mmol/L]	26.3 ± 0.4	51.9 ± 1.1	76.7 ± 1.6	13.6-15.7 ^{99,100}
c(GML) [mmol/L]	31.8 ± 0.6	64.3 ± 1.3	97.6 ± 1.9	0.044 ¹⁰¹
c(C12E10) [mmol/L]	13.6 ± 0.3	27.5 ± 0.5	41.7 ± 0.8	0.09-0.14 ^{102,103}
c(SLEC) [mmol/L]	17.0 ± 0.3	33.4 ± 0.6	49.3 ± 0.9	~0.4 (at pH 7) ^{91,104}
c(NaSal) [mmol/L]	50.7 ± 1.0	97.3 ± 1.9	147.4 ± 2.8	-

In the present case, surface-active molecules rather than classical electrolytes were introduced to the system. Therefore, the possibility of self-association under the given conditions is briefly discussed below. The concentrations used, the CMCs of the surfactants (see Table 4.1), the effects of EtOH and Oct on the CMCs, and mesostructuring are considered. The concentrations used exceed the CMCs of the surfactants in pure water. Considering the presence of EtOH and Oct, contrasting factors affecting the CMC must be discussed. Although the exact values depend on the surfactant, EtOH is widely recognized to decrease the CMC at low $c(\text{EtOH})$ in binary EtOH-water mixtures and to increase it above a certain concentration. By lowering the dielectric constant of water and thus weakening the hydrophobic effect in bulk water, the driving force for micelle formation is reduced. EtOH molecules can also partly partition into the palisade region of the micelles and act as a cosolvent, stabilizing the micelles at low $c(\text{EtOH})$. At higher EtOH levels, their penetration into the micelles loosens interfacial packing and, combined with the reduced hydrophobic effect, progressively destabilizes the micelles^{97,100,105–108}. Above around 40% (v/v) EtOH, micellization is reported to be completely suppressed¹⁰⁹. Oct, on the other hand, is considered a classical cosurfactant. It facilitates micellization by penetrating micelles and increasing hydrophobic interactions^{106,107,110–112}. Other critical parameters affected by additives are the surfactant aggregation number N_{Agg} and the degree of micelle ionization α . N_{Agg} describes the number of surfactants per micelle. It decreases upon the addition of short-chain alcohols and increases upon the addition of medium-chain alcohols. α describes the degree of counterion dissociation and increases upon the addition of short-chain alcohols^{97,112}.

Unlike binary or ternary molecular solvent mixtures, SFMEs exhibit different mesostructures with highly fluctuating interfaces and pseudo-phases (see Figure 4.2). To understand how the components behave in solution, their solubility in the system should also be considered. The amounts of nonionic surfactants C12E10 and GML, as well as SLEC and NaSal, could be fully solubilized in EtOH. The presence of EtOH in all pseudo-phases suggests that their interfacial affinity might be reduced. The ionic surfactants SDS, NaL, and LTAB could not be fully solubilized in EtOH and may have a higher affinity for the interface. The points examined in the phase diagrams exhibit a constant EtOH amount of 31-40% (w/w) and varying Oct and water amounts (10-40% and 20-59% (w/w), respectively), with an increasing Oct proportion along the binodal. The significant reduction in the binodal upon adding ionic compounds implies the solubilization of Oct in water as the water proportion increases and the EtOH proportion decreases. This could either happen by incorporating Oct into existing mesostructures or by molecular solubilization in the water-EtOH bulk phase. The latter option could occur only if the solvent polarity of water were significantly reduced. Given the significant concentration difference between the additive and water, this is not probable. Considering the factors discussed above, it can only be speculated about the behavior of the individual compounds and their influence on the mesostructure. DLS and SAXS measurements were conducted to gain better insight.

4.4.2. The Impact of Surface-active Compounds on the Mesostructuring of EtOH/Oct/Water

4.4.2.1. Comparison of DLS Apparent Coherence Factors β and SAXS Forward Scattering $I(q \rightarrow 0)$

DLS and SAXS measurements were performed at specific intervals based on the Oct content (indicated by the symbols in Figure 4.5) and at various fixed EtOH (+compound) levels. This means that the measurement points move into the former Ouzo region as the binodal decreases. DLS data are provided as intensity autocorrelation functions $g^{(2)}(\tau)-1$ and normalized field autocorrelation functions $g^{(1)}(\tau)$ in Figure SI 4 1. DLS and SAXS fit parameters are provided in Table SI 4 1 and Table SI 4 2, respectively. The apparent coherence factors β obtained from cumulant fitting of $g^{(2)}(\tau)-1$ and the forward scattering intensities $I(q \rightarrow 0)$, derived from fitting SAXS data to a combined Ornstein-Zernike (OZ) and broad peak (BP) model, are presented in Figure 4.7. β is a sample-dependent measure of the proportion of coherent scattering intensity and can be approximated from the y-axis intercept of the correlation function. Depending on the SAXS data fitting range, the low- q range is sometimes described by the OZ contribution as an upturn, resulting in unreasonably high OZ-related values, as indicated in Figure 4.6 **A1**. This case was excluded, and fitting was performed as indicated in **A2**.

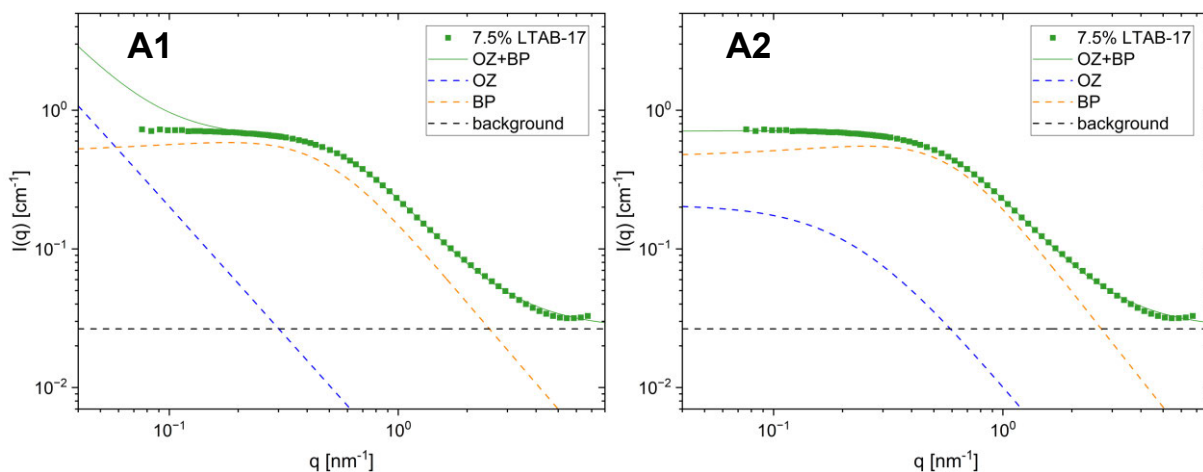


Figure 4.6: OZ contribution at low q depending on the fitting range. Case **A1** produces an upturn of the OZ+BP fit and results in unreasonably high OZ fit parameters. Case **A2** describes the SAXS data well. The green line indicates the combined fit (OZ+BP(+background)), the blue dashed line indicates the OZ contribution, the orange dashed line indicates the BP contribution, and the black dashed line indicates the flat background contribution.

The majority of the compositions studied in the former biphasic region (except for 40% Oct of the pure SFME, with nonionic surfactants, and with 2.5% NaSal) exhibit pronounced apparent coherence factors β (Figure 4.7 **A1-G1**), indicating structuring throughout the entire area, not just near the pseudo-critical point^{113,114}. β is generally higher with the presence of additives compared to pure SFME. While there is a clear relationship between the increase in β and additive concentration for GML and, less

pronounced but visible, for C12E10 between 10 to 32% Oct (EtOH(black)<2.5%(red)<5%(blue)<7.5%(green)), this relationship is less obvious for ionic surfactants at 10-24% Oct and becomes more evident again at 32 and 40% Oct. The addition of NaSal likely produces a trend similar to that of nonionic surfactants, but the high β values for 5% NaSal, along with noticeably lower decay rates Γ at 10-24% Oct (see Table SI 4 1), are likely due to the samples being measured too close to the binodal. Therefore, they are excluded from the evaluation. For nonionic surfactants and NaSal, the absolute increase in β compared to the pure SFME is larger at 10-24% Oct and then decreases at 32 and 40% Oct. For ionic surfactants, it is larger at 17-32% Oct and smaller at 10% Oct. At 40% Oct, increasing β values are observed from nonionic surfactants and NaSal via SLEC to ionic surfactants. The behavior of β aligns with the different positions of the measurement points relative to the binodal. As shown in Figure 4.5, the shape of the binodal changes when additives are present. To keep $c(\text{EtOH}+\text{compound})$ constant, the measurement points vary in their distance from the binodal. This could explain the fluctuations in the absolute β values. The main point is that β generally increases when additives are present, except at 40% Oct when nonionic surfactants are added.

For the addition of ionic surfactants, the highest β values shift toward higher Oct ratios (24-32% Oct), compared to 17% Oct in the pure SFME. This shift becomes more significant with increasing concentration. In contrast, the addition of nonionic surfactants and NaSal shows the highest β values at 17% Oct, regardless of additive concentration. The results for NaL are again consistent with those from preliminary experiments with SDS by Jonas Blahnik⁹⁰. The decay rates Γ generally decrease when different additives are added to the pure SFME (see Table SI 4 1). This indicates a change in the structuring, caused by changes in aggregate structure and/or size, rather than pure stabilization of the present structures.

The forward scattering intensities $I(q \rightarrow 0)$ confirm the presence of scattering entities throughout the entire analyzed region (**A2-G2**). Generally, $I(q \rightarrow 0)$ increases with the addition of compounds compared to pure SFME. The way $I(q \rightarrow 0)$ rises, reflecting its dependence on the compound's concentration and overall intensity behavior, generally aligns with results from DLS measurements. High $I(q \rightarrow 0)$ values at 5% NaSal also suggest an insufficient distance to the binodal, as indicated by DLS data. The shifts of most intense structuring toward higher Oct proportions upon adding ionic surfactants are also clear in the SAXS data. The concentration dependence of this effect is more prominent than that of β . The only exception is NaSal: while β values show no shift, the maximum $I(q \rightarrow 0)$ increases from 17% Oct to 24% Oct as $c(\text{NaSal})$ rises from 2.5% to 7.5%.

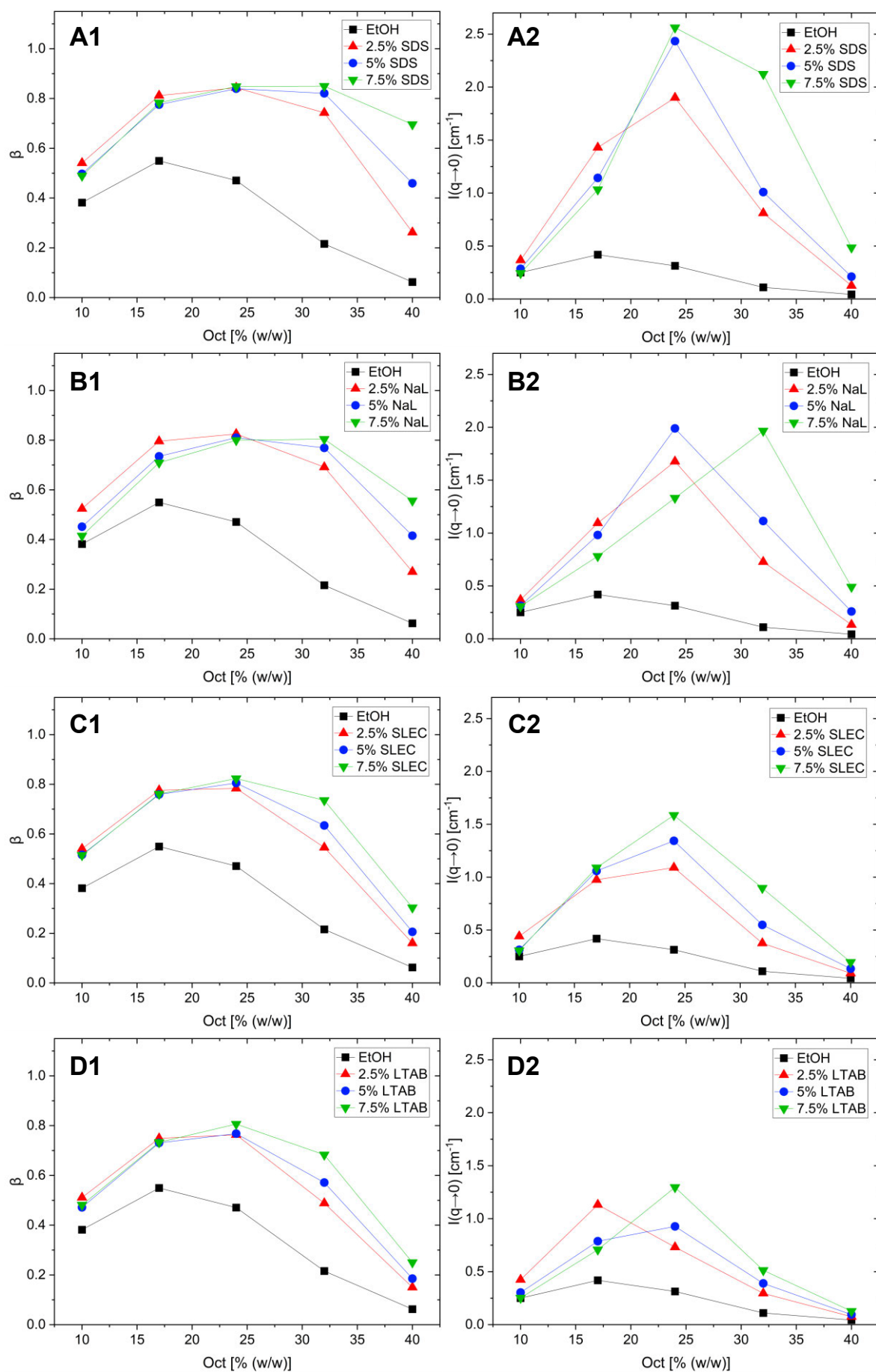


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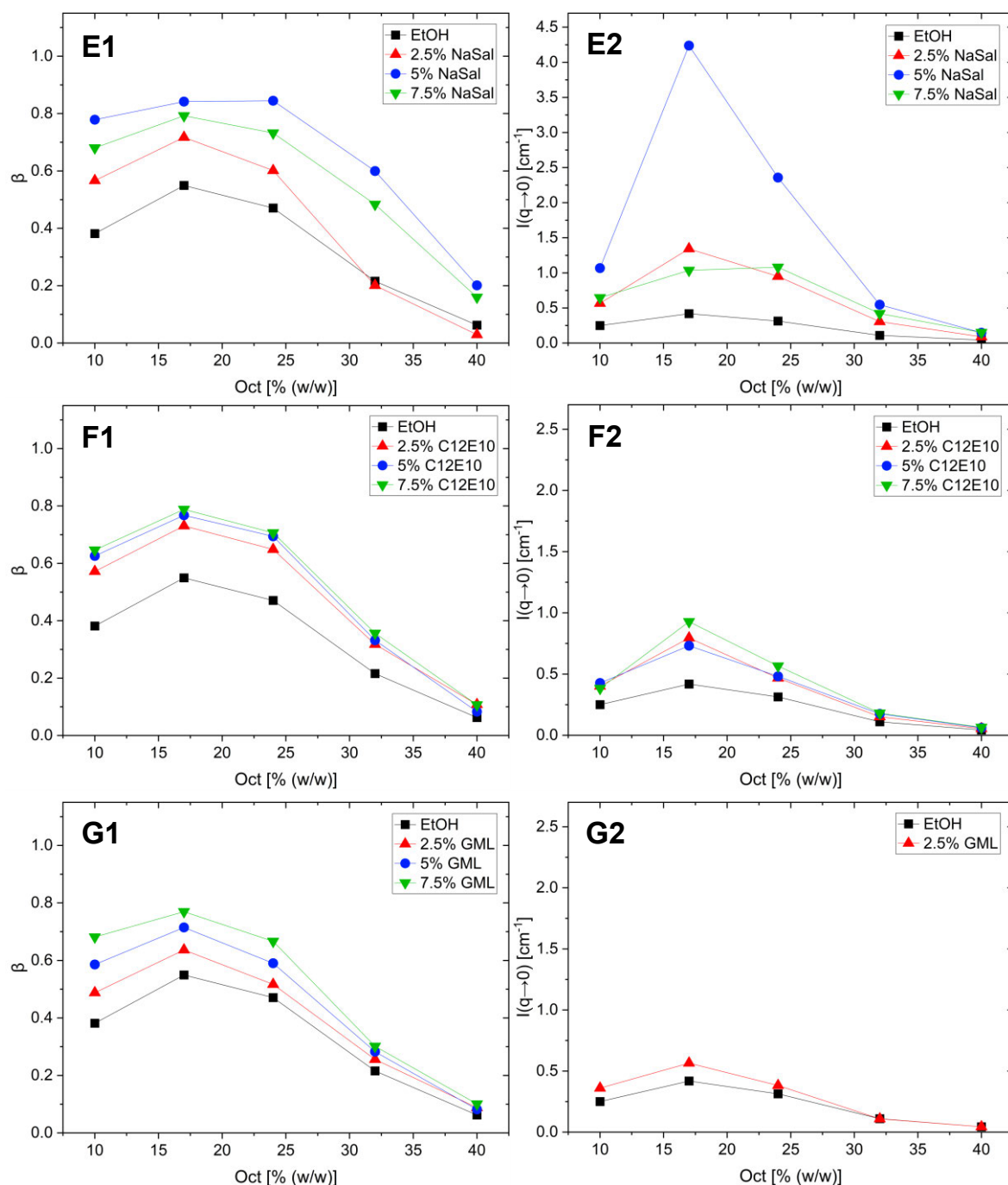


Figure 4.7: Apparent coherence factors β obtained from second-order cumulant fitting of DLS intensity autocorrelation functions $g^{(2)}(\tau)-1$ (**A1-G1**) and forward scattering intensities $I(q \rightarrow 0)$ obtained from a combined Ornstein-Zernike (OZ) and broad peak (BP) fit model to the SAXS data (**A2-G2**) in pure EtOH/Oct/Water and with the addition of 2.5-7.5% compound SDS (**A**), NaL (**B**), SLEC (**C**), LTAB (**D**), NaSal (**E**), C12E10 (**F**), and GML (**G**). SAXS data for 5% and 7.5% GML were not evaluated due to impurities. Model parameters are provided in Table SI 4 1.

When comparing β values with $I(q \rightarrow 0)$, certain considerations must be taken into account: $g^{(2)}(\tau)-1$ describes autocorrelated intensity fluctuations over time, while forward scattering reflects an absolute intensity from a static X-ray scattering experiment (see general information on scattering techniques in chapter 1.2.3). The low β values of the pure SFME (0.12-0.65) indicate the highly dynamic and polydisperse nature of the present aggregates: due to numerous superimposed mesoscopic fluctuations that are not coherent with each other or are weakly scattering, resulting in a low signal-

to-noise ratio, the overall intensity is only weakly correlated, and β values are not close to 1¹¹⁵. An increase in β upon adding surfactants or NaSal could indicate the formation of more coherent fluctuations and thus less polydisperse structures, more stable structures with longer lifetimes, or more defined structures, creating better contrast. Changes in Γ indicate changes in the aggregate structure. $I(q \rightarrow 0)$ is proportional to the contrast, the concentration of the scatterer, and the volume of the scatterer (see equation 1.15)¹¹.

At this stage, it can only be speculated about how the contrast changes with the addition of surfactants and NaSal. A change in the concentration of scatterers would imply either the formation of new scatterers upon adding the compounds or a transformation of the mesostructure, especially the pre-Ouzo aggregates. Since the solvent proportions are unchanged, the transformation of mesostructures would directly relate to their size. However, this cannot be estimated from $I(q \rightarrow 0)$ alone; it requires analysis of the entire scattering profile.

4.4.2.2. Structural Interpretation of SAXS Profiles Using a Combined Ornstein-Zernike and Broad Peak Fit Model

To analyze the systems in more detail, SAXS data were fitted using a model consisting of two uncorrelated Lorentzian functions (see equation 4.4). Prévost *et al.* originally introduced this model to fit SAXS data of EtOH/Oct/Water across the entire monophasic region in the phase diagram³⁷. The OZ contribution is typically used to model critical fluctuations without a defined interface and has been widely applied to fit data of pre-Ouzo aggregates^{36,55,116}. The BP contribution is often used for structures with repeating characteristic distances, such as in amorphous structures³⁷. In the case of SFMEs, it was found that the BP contribution effectively describes the clustering of hydroxy groups in the Oct bulk phase³⁷. By linking the two contributions, the fit model accounts for both pseudo-phases without considering a form factor for structures³⁷.

Scattering profiles and fits of pure SFME, along with two representative cases, SDS and C12E10, and NaSal, are shown in the following figures and discussed in detail. The SAXS profiles of NaL, SLEC, LTAB, and GML have been prepared similarly and are available in Figure SI 4 3-6. Two different representations of the scattering data of EtOH/Oct/Water across the binodal are illustrated in Figure 4.8. On a log-log scale (**A1**), the entire scattering profile is clearly visible, despite the intensities covering several orders of magnitude. Power laws ($I \propto q^{-n}$), here $I \propto q^{-1}$, are easily recognizable. On a lin-lin scale (**A2**), subtle changes in the low q range, like the development from kinks to peaks, are more apparent than in a log-log scale.

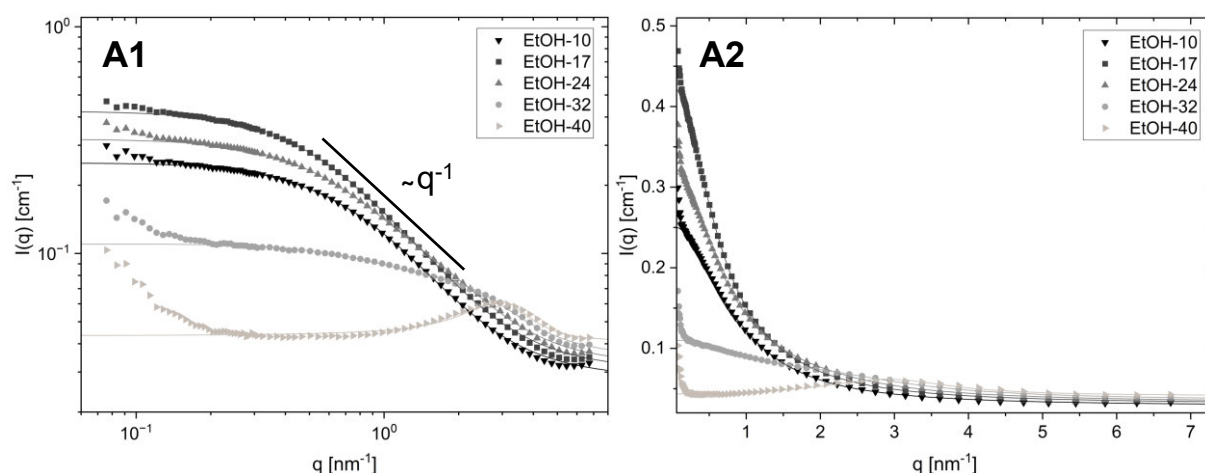


Figure 4.8: Scattering profiles of EtOH/Oct/Water with varying concentrations of Oct in log-log scale (**A1**) and lin-lin scale (**A2**). Symbols are SAXS data and solid lines are OZ+BP-fitted curves. Model parameters are provided in Table SI 4 2. Scattering data decay with $\sim q^{-1}$.

The scattering data of the pure SFME follow a classical OZ-like behavior at 10-24% Oct. This means that no structure factor peak is visible in the scattering profile, the intensity reaches a plateau at low q , and it decays with $\sim q^{-2}$ toward high q ¹¹. Due to the overall low intensity in this case, the combined fit contributions of OZ and the flat background produce an apparent q^{-1} decay. At 32% and 40% Oct, a structure factor peak appears around 3 nm^{-1} , caused by clusters of hydroxy groups from Oct molecules (Oct-OH clusters). In pure Oct, the peak appears at 4.1 nm^{-1} , indicating a distance of 1.5 nm between Oct-OH clusters. When water or EtOH is added, this peak shifts to lower q -values, and the distance between Oct-OH clusters increases^{28,37,117}. Based on the composition of the samples and the example scattering profiles explained by Lopian *et al.*²⁸ and Prévost *et al.*³⁷, it can be assumed that samples at 10-24% Oct are in the pre-Ouzo region, the sample at 32% Oct marks the transition to the BIC region, and that at 40% Oct marks the transition to the oil-rich/reverse aggregate region.

The scattering profiles of (EtOH+SDS)/Oct/Water with 2.5-7.5% SDS are shown in Figure 4.9. It can be observed that the increase in forward scattering cannot be explained solely by a change in contrast, as the scattering profiles are also changing. Analyzing the course along the binodal, a structure factor peak appears in the Oct-poor region (10-17% Oct). As the Oct proportion increases, the scattering profiles exhibit more OZ-like behavior (24-32% Oct). At 40% Oct, the peak from the Oct bulk phase is visible around $3\text{-}4 \text{ nm}^{-1}$ and is less pronounced than in the pure SFME. Since the peak is barely visible on a lin-lin scale, the data range has been restricted to better examine the relevant low- q data. All scattering profiles with 2.5-7.5% SDS follow the same pattern, but peaks are more prominent and extend across a broader range of Oct with higher $c(\text{SDS})$. At 40% Oct, the OZ contribution increases with increasing $c(\text{SDS})$. It is important to emphasize that the fit model includes only one Lorentzian function with a non-zero mode that produces peaks. As discussed above, the peak produced by Oct-OH clusters around $2\text{-}4 \text{ nm}^{-1}$ shifts toward lower q with increasing amount of water^{28,37,117}. The maximum of the structure factor peak at low q for small Oct amounts when adding SDS shifts first to

smaller q and then back toward higher q as the Oct amount increases. The shift of the BP contribution in SAXS profiles without a clearly visible peak is visualized in Figure SI 4 2. The influence of both structural motifs on the scattering pattern changes gradually, but only one peak is captured by the fit. Therefore, the more prominent one is considered. Adding another non-zero Lorentzian function to the model would solve this issue, but it would also make the fit less stable.

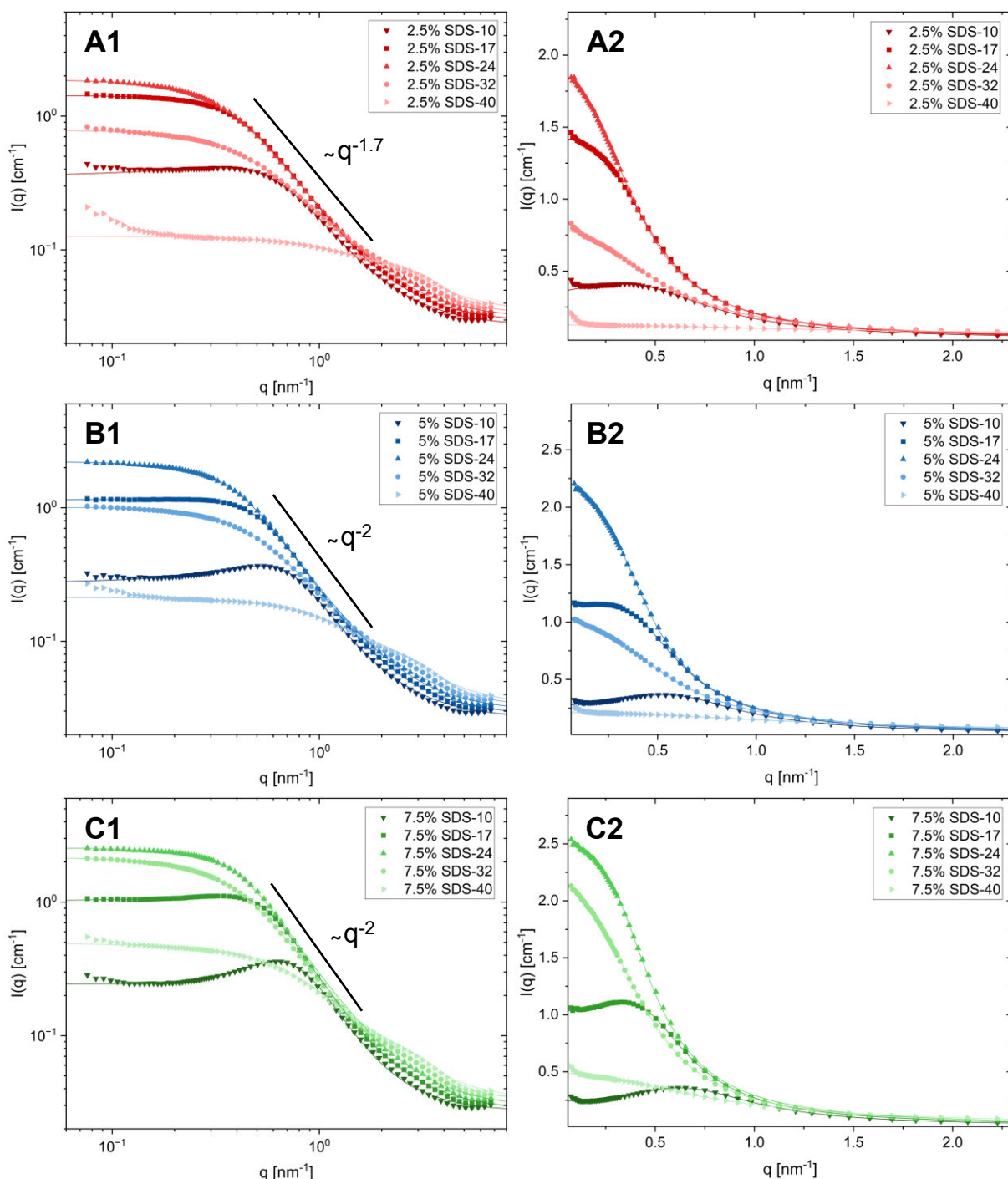


Figure 4.9: Scattering profiles of EtOH+SDS/Oct/Water with varying concentrations of Oct (10-40%) in log-log scale (A1-C1) and lin-lin scale (A2-C2). Symbols are SAXS data and solid lines are OZ+BP-fitted curves. Model parameters are provided in Table SI 4 2. Scattering data decay with $\sim q^{-2}$.

The evolution of scattering data at distinct points (% Oct) in the phase diagram, as SDS is added, is summarized in Figure 4.10. At 10% Oct (**A**), the pure SFME exhibits OZ-like behavior, with a structure factor peak appearing and shifting toward higher q as $c(\text{SDS})$ increases (from 0.3 nm^{-1} at 2.5% SDS to 0.6 nm^{-1} at 7.5% SDS). At 17% Oct (**B**), the peak disappears at lower $c(\text{SDS})$ and only remains at higher $c(\text{SDS})$. At 24% Oct (**C**), no peaks are visible, but a shoulder at 7.5% SDS remains, which also disappears at 32% Oct (**D**). The scattering profiles exhibit OZ-like behavior with a q^{-2} decay due to increased intensity. At 32% Oct, the shoulder at 7.5% SDS disappears, and the peak of pure Oct appears. At 40% Oct (**E**), the peak of pure Oct is clearly visible in the pure SFME but is superimposed by the OZ contribution as $c(\text{SDS})$ increases. All scattering profiles from 2.5 to 7.5% SDS exhibit the same behavior, with peaks becoming more pronounced and extending across a broader range of Oct as $c(\text{SDS})$ increases. The scattering patterns indicate the appearance of a structure factor at low Oct amount with increasing $c(\text{SDS})$. Additionally, at 32% Oct (**D**), the scattering patterns shift from a BIC-like pattern to a more pre-Ouzo-like pattern. At 40% Oct (**D**), the patterns gradually change from an oil-rich pattern to a BIC-like pattern, and then to a pre-Ouzo-like pattern as $c(\text{SDS})$ increases. Therefore, greater stabilization of pre-Ouzo aggregates is suggested, leading to the gradual suppression of reversed aggregates and, if a sufficiently high amount of surfactant is added, the BIC phase.

Considering the addition of surfactants to the system and the appearance of structure factor peaks in the low q region when ionic surfactants are added, the formation of micelles should be considered. Pure SDS micelles display a structure factor peak at $q = 1.6 \text{ nm}^{-1}$ in water^{118,119}. Since all tested anionic surfactants have a C12 chain, a similar peak position is expected for NaL and LTAB micelles. SLEC micelles are estimated larger^{91–93}. The peaks discussed here emerge around 0.5 nm^{-1} and shift toward higher q -values (around 0.8 nm^{-1}) with increasing $c(\text{surfactant})$. Blahnik presented SAXS data of EtOH(+SDS)/Oct/Water at a specific point in the pre-Ouzo region and in the former Ouzo region at SDS concentrations between 1.0% ($\approx 11 \text{ mmol/L}$) and 7.3% ($\approx 80 \text{ mmol/L}$). He also observed a shift toward higher q -values with increasing $c(\text{SDS})$ (from 0.8 nm^{-1} to 1.2 nm^{-1}) and suggested the formation of mixed micelles⁹⁰. Marcus *et al.* observed peak formation at intermediate q in the scattering patterns upon the addition of SDS. They added SDS at concentrations between 0.07 and 0.55 mol/kg, corresponding to 0.06 mol/L and 0.48 mol/L, respectively, taking into account the sample composition, and attributed the emerging peak to the progressive formation of mixed micelles⁵⁵. Direct comparison of $I(q)$ is difficult, as Blahnik and Marcus *et al.* added SDS to a constant composition without accounting for the reduction in the binodal. In the present case, the measuring points were adjusted to have a constant distance from the binodal.

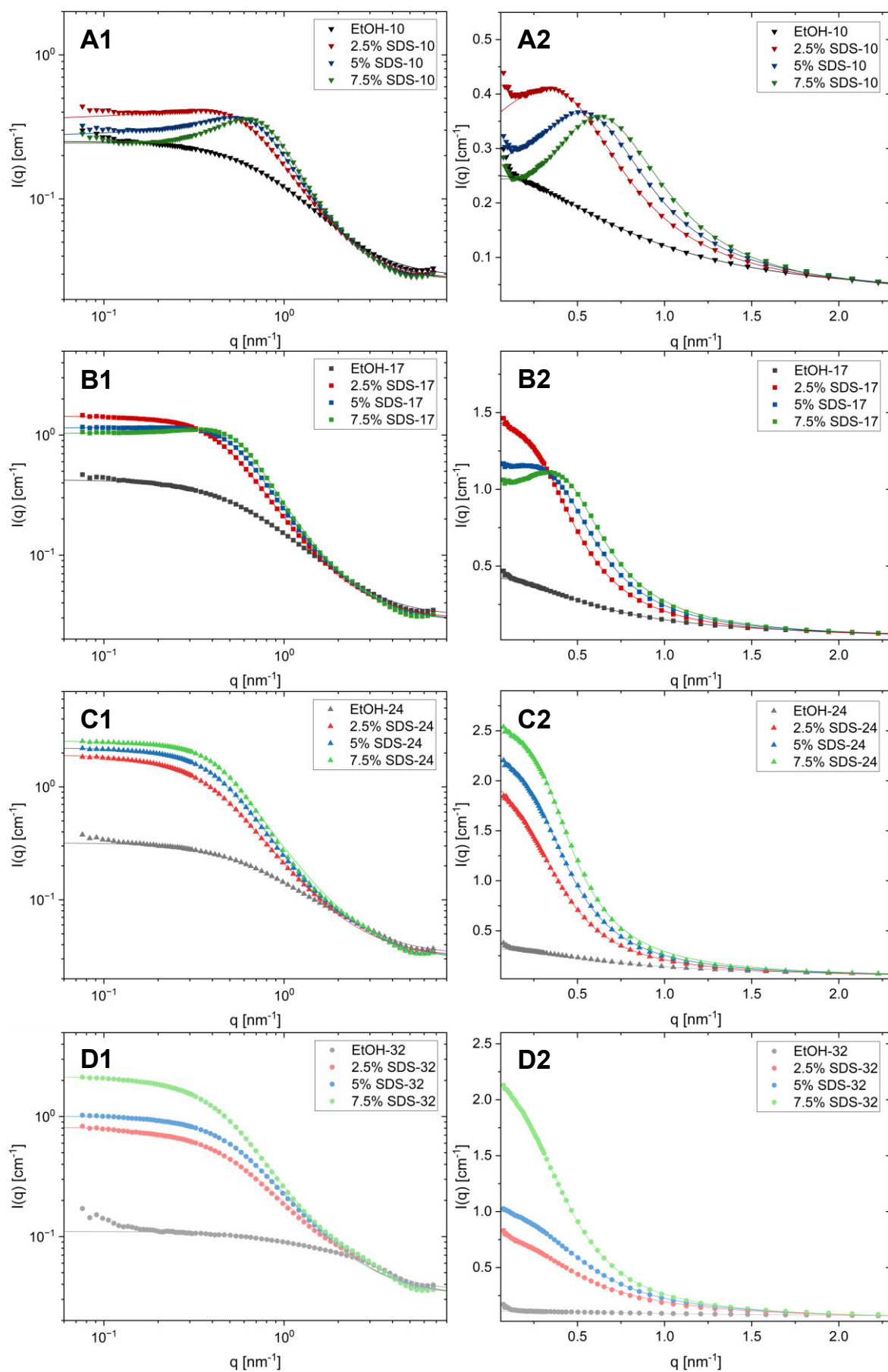


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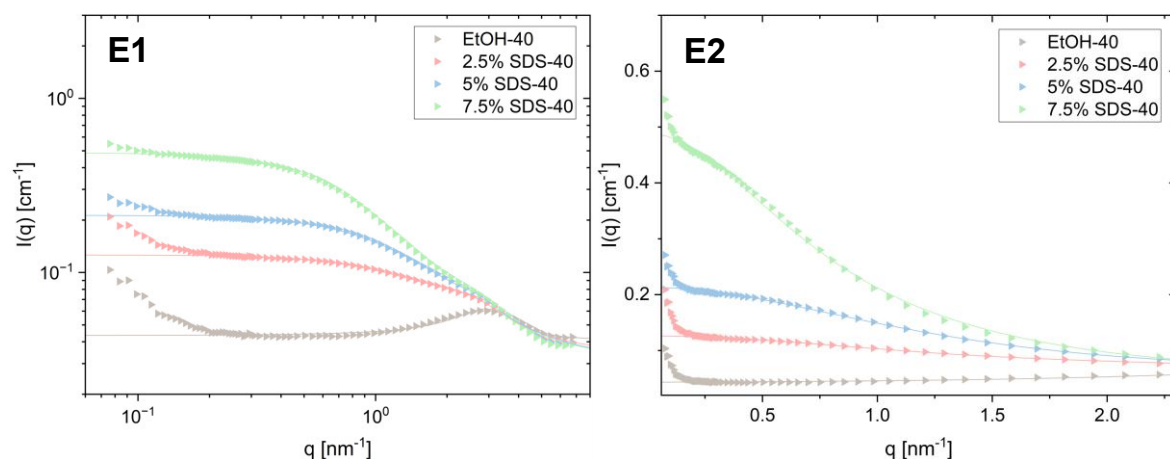


Figure 4.10: Scattering profiles of EtOH(+SDS)/Oct/Water at distinct % Oct with increasing $c(\text{SDS})$ in log-log scale (**A1-E1**) and lin-lin scale (**A2-E2**). Symbols are SAXS data and solid lines are OZ+BP-fitted curves. Model parameters are provided in Table SI 4 2.

Given the significantly lower SDS concentration here, it can be assumed that the system consists of Oct droplets with SDS molecules at the interface, rather than micelle-like structures. At 10% Oct, electrostatic effects could also be present. As the SDS concentration remains constant across all samples, at higher % Oct, electrostatic effects should decrease due to a lower surfactant-to-Oct-ratio. The sample at 10% Oct probably represents a preliminary state of the system's behavior observed by Blahnik⁹⁰ and then, with further increases in $c(\text{SDS})$, by Marcus *et al.*⁵⁵. The latter speculated about the behavior upon the addition of very low $c(\text{SDS})$ and considered the destruction of pre-Ouzo aggregates at very low $c(\text{SDS})$. The experiments presented here could disprove this hypothesis.

The addition of NaL (see Figure SI 4 3) affects the data similarly to SDS. With 2.5% NaL, a flat peak at 10% Oct and a prominent shoulder at 17% Oct are observed. The shoulder develops into a peak as $c(\text{NaL})$ increases, and a shoulder at 24% Oct appears with 5% and 7.5% NaL. The scattering profiles show more OZ-like behavior as the Oct proportion rises. At 40% Oct, the peak from the Oct bulk phase appears around $3\text{--}4\text{ nm}^{-1}$ and is superimposed by the OZ contribution as $c(\text{NaL})$ increases. The evolution of the scattering data at different points follows the same pattern as with SDS. Unlike SDS, the peaks at 10% Oct obtained with NaL show a distinct upturn at low q , while those produced by SDS reach a plateau. The increasing intensity at low q at 32% and 40% Oct with 5% NaL, and at 40% Oct with 7.5% NaL, may indicate measurements taken too close to the binodal.

SLEC and LTAB (see Figure SI 4 4 and Figure SI 4 5, respectively) affect the scattering behavior of EtOH/Oct/Water similarly to SDS and NaL, but to a lesser extent. When SLEC or LTAB is added, a structure factor peak at 10% Oct appears, becoming more prominent as $c(\text{SLEC/LTAB})$ increases, although its shift toward higher q is less pronounced than with SDS. No clear peak appears at 17% Oct; only shoulder formation occurs with increasing $c(\text{SLEC/LTAB})$. The scattering profiles show more OZ-like behavior as the Oct proportion grows. Similar to SDS and NaL, the peak from the Oct bulk phase appears around $3\text{--}4\text{ nm}^{-1}$ but is superimposed by OZ at higher $c(\text{SLEC})$. In the case of SLEC, the earlier

noted concentration difference (see Table 4.1) should be considered when comparing the less distinct scattering profiles to those from SDS.

Upon adding NaSal, no peaks appear regardless of $c(\text{NaSal})$ (see Figure 4.11) and scattering profiles decay with $\sim q^{-1.5}$. The relatively high increase observed with 5% NaSal, already discussed in the context of Figure 4.6, is due to a too small distance to the binodal. The evolution of scattering data at distinct points (% Oct) in the phase diagram, as NaSal is added, is summarized in Figure 4.12. At 10-32% Oct (**A-D**), scattering patterns show OZ-like behavior. At 40% Oct (**E**), the scattering patterns shift from an oil-rich pattern, as indicated in the pure SFME at that % Oct, to a BIC-like pattern regardless of $c(\text{NaSal})$. Similar to the addition of SDS, stabilization of pre-Ouzo aggregates is suggested at low to intermediate Oct proportion. The BIC phase appears to be stabilized at a high Oct proportion, suppressing the formation of reverse aggregates. NaSal seems to act similarly but less pronouncedly than the ionic surfactants. This is also indicated by the overall higher $I(q)$ upon the addition of surfactants. The solubility of the hydrotrope in EtOH is higher than that of the ionic surfactants. NaSal probably dissolves better in both pseudo-phases and is thus less affine for the interface. Therefore, the stabilizing effect might follow a similar mechanism but might be less pronounced than with surfactants.

The impact of charged hydrotropes on the SFME mesostructure has rarely been studied in the literature^{90,120,121}. El Maangar *et al.* studied the mesostructure of the system NaSal/Ethyl acetate/Water. They found dynamic aggregates of NaSal and ethyl acetate near the liquid-solid phase boundary, as well as micelle-like structures of NaSal in water saturated with ethyl acetate above a NaSal concentration of 1 M¹²⁰. They referred to the structure as similar to the ones observed by Marcus *et al.*⁵⁵ upon the addition of antagonistic salts. Marcus *et al.* studied the influence of antagonistic salts on EtOH/Oct/Water and observed stabilization of pre-Ouzo aggregates caused by charging of the interface. This was evident from a decrease in $I(q \rightarrow 0)$ and simultaneous peak formation at intermediate q ⁵⁵. Referring to the findings of Sadanake *et al.*^{122,123} and Onuki *et al.*⁵⁶, the antagonistic ion acts as a surface-active compound, creating a diffuse interface stabilized by pure electrostatics⁵⁵. As discussed with the addition of SDS, the concentrations used in the presented case are far below those reported in the literature; thus, no repulsive structures are observed, and only a slight charging and stabilization of mesostructures are proposed.

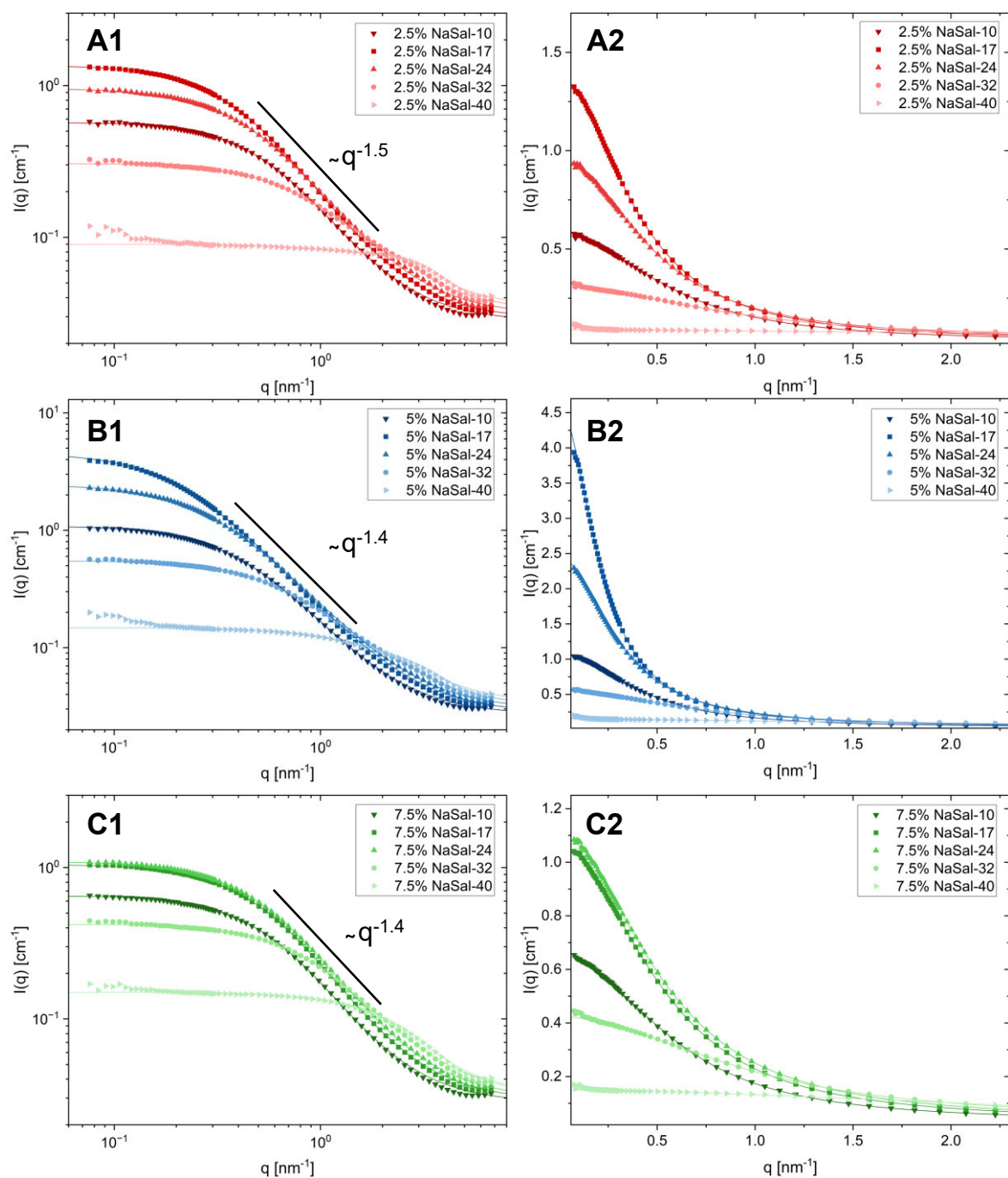


Figure 4.11: Scattering profiles of EtOH+NaSal/Oct/Water with varying concentrations of Oct (10-40%) in log-log scale (**A1-C1**) and lin-lin scale (**A2-C2**). Symbols are SAXS data and solid lines are OZ+BP-fitted curves. Model parameters are provided in Table SI 4 2. Scattering data decay with $\sim q^{-1.5}$.

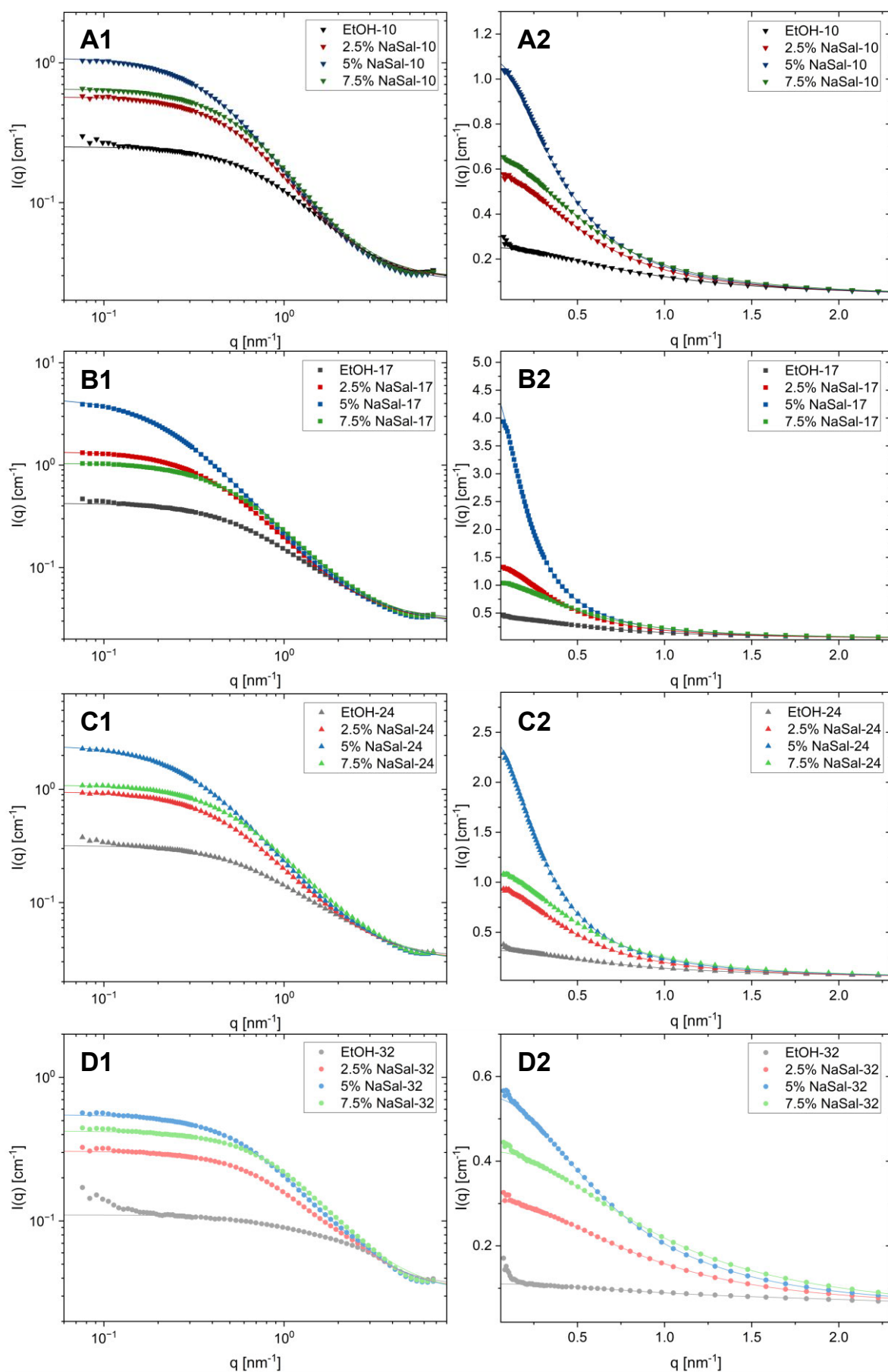


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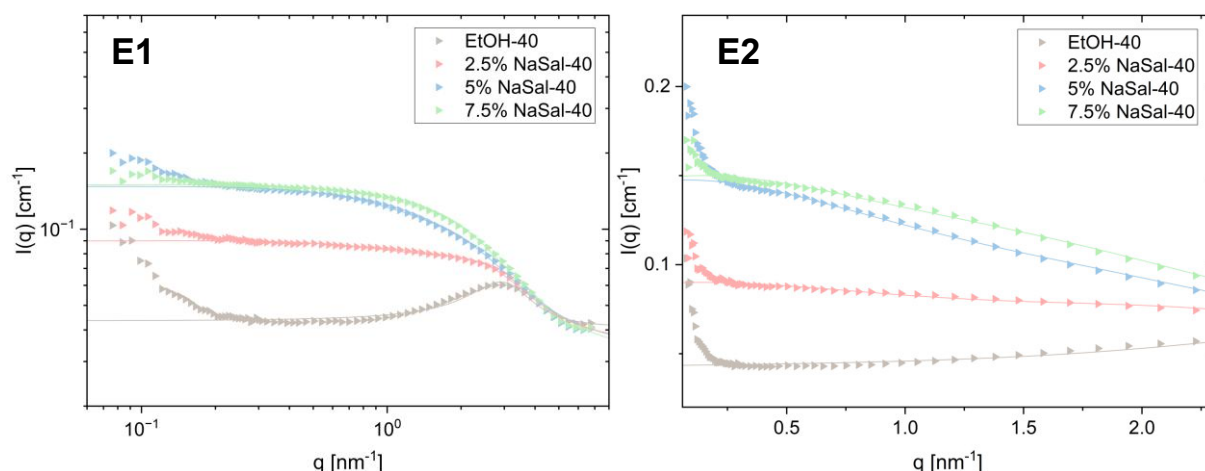


Figure 4.12: Scattering profiles of EtOH(+NaSal)/Oct/Water at distinct % Oct with increasing $c(\text{NaSal})$ in log-log scale (**A1-E1**) and lin-lin scale (**A2-E2**). Symbols are SAXS data and solid lines are OZ+BP-fitted curves. Model parameters are provided in Table SI 4 2.

The scattering profiles of C12E10 are shown in Figure 4.13 and Figure 4.14. Examining the trend along the binodal (Figure 4.13), all scattering profiles display OZ-like behavior, with no peaks observed at 10–24% Oct. At 32–40% Oct, the pure Oct structure factor peak appears around 3 nm^{-1} . At specific points in the phase diagram (% Oct), the scattering patterns remain largely unchanged with the addition of 2.5–7.5% C12E10 compared to the pure SFME (Figure 4.14). A change occurs at 40% Oct: as $c(\text{C12E10})$ increases, the scattering pattern shifts toward a more BIC-like pattern, indicating a slight stabilization of that mesostructure upon adding C12E10.

Upon adding GML, no peaks appear in the scattering profiles (see Figure SI 4 6). At 2.5% GML, no changes occur compared to the pure SFME. At 5% and 7.5% GML, noticeable low- q upturns are visible across all compositions in the scattering profiles. Measurements taken too close to the binodal can be excluded, as indicated by the course of the binodal (see Figure 4.5). Possible reasons for the observed patterns include temperature-induced phase transition during sample preparation or impurities in the samples (purity of GML $\approx 90\%$). DLS samples were filtered prior to measurement, and the correlation functions are similar to those observed in other cases. Samples were not filtered before SAXS measurements, and potential impurities were not removed. Based on the DLS and SAXS data for 2.5% GML, the latter explanation is more probable. Therefore, the SAXS profiles of 5% and 7.5% GML were not further evaluated.

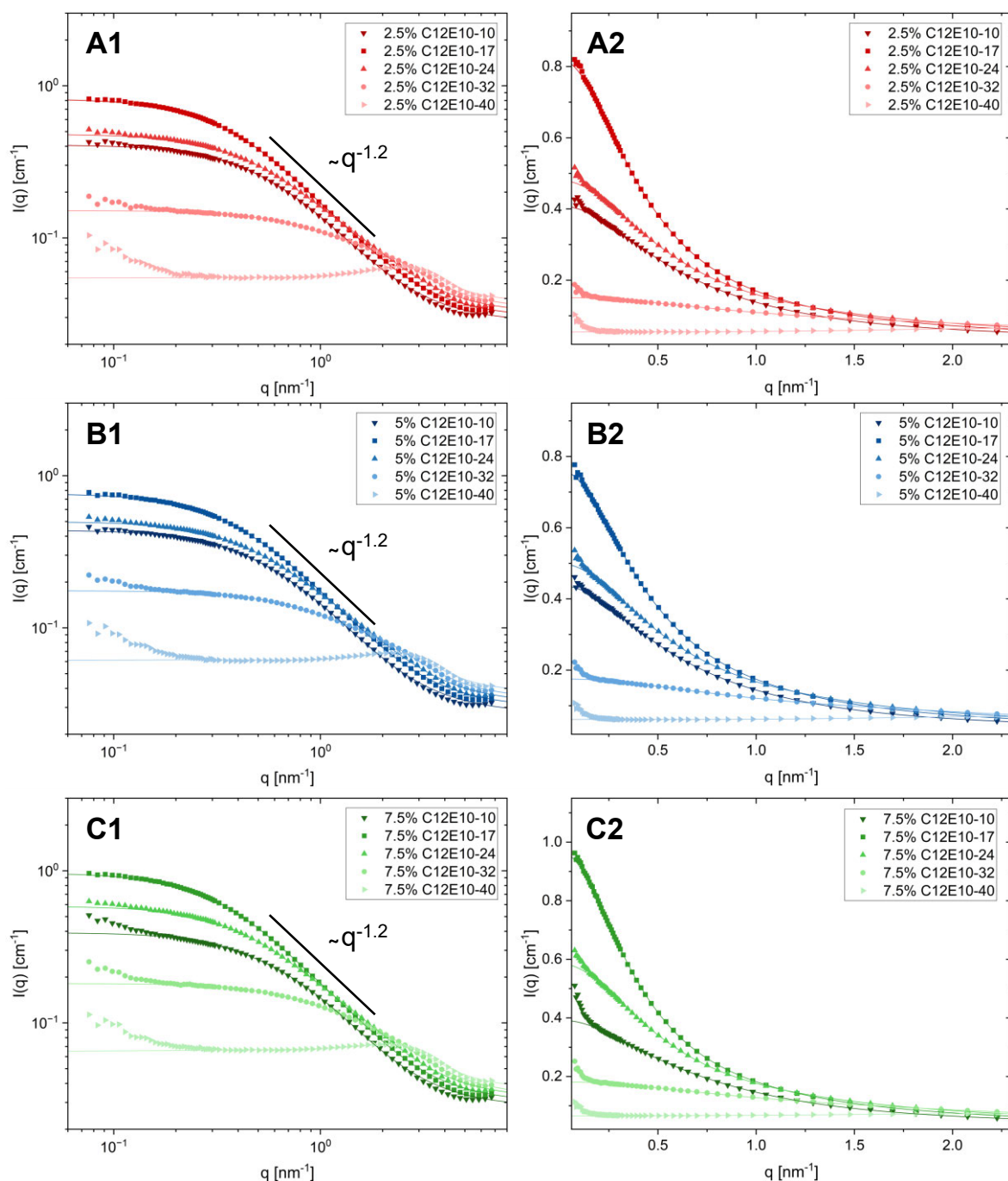


Figure 4.13: Scattering profiles of EtOH+C12E10/Oct/Water with varying concentrations of Oct (10-40%) in log-log scale (**A1-C1**) and lin-lin scale (**A2-C2**). Symbols are SAXS data and solid lines are OZ+BP-fitted curves. Model parameters are provided in Table SI 4 2. Scattering data decay with $\sim q^{-1.2}$.

The evolution of scattering data at specific points along the binodal, compared to points in the pure SFME, indicates changes in the mesostructure with the addition of ionic surfactants. More defined structures are formed at low % Oct, and the pre-Ouzo region seems to be enlarged, inducing a shift of the BIC structure toward higher % Oct. The scattering data follow different power laws with stronger decay as ionic compound concentration increases. However, no Porod law ($I(q) \sim q^{-4}$), as expected for sharp interfaces¹²⁴, is observed. This indicates that the structures are more defined and less fluctuating than for classical SFMEs, but less defined and more fluctuating than for classical microemulsions^{11,37}.

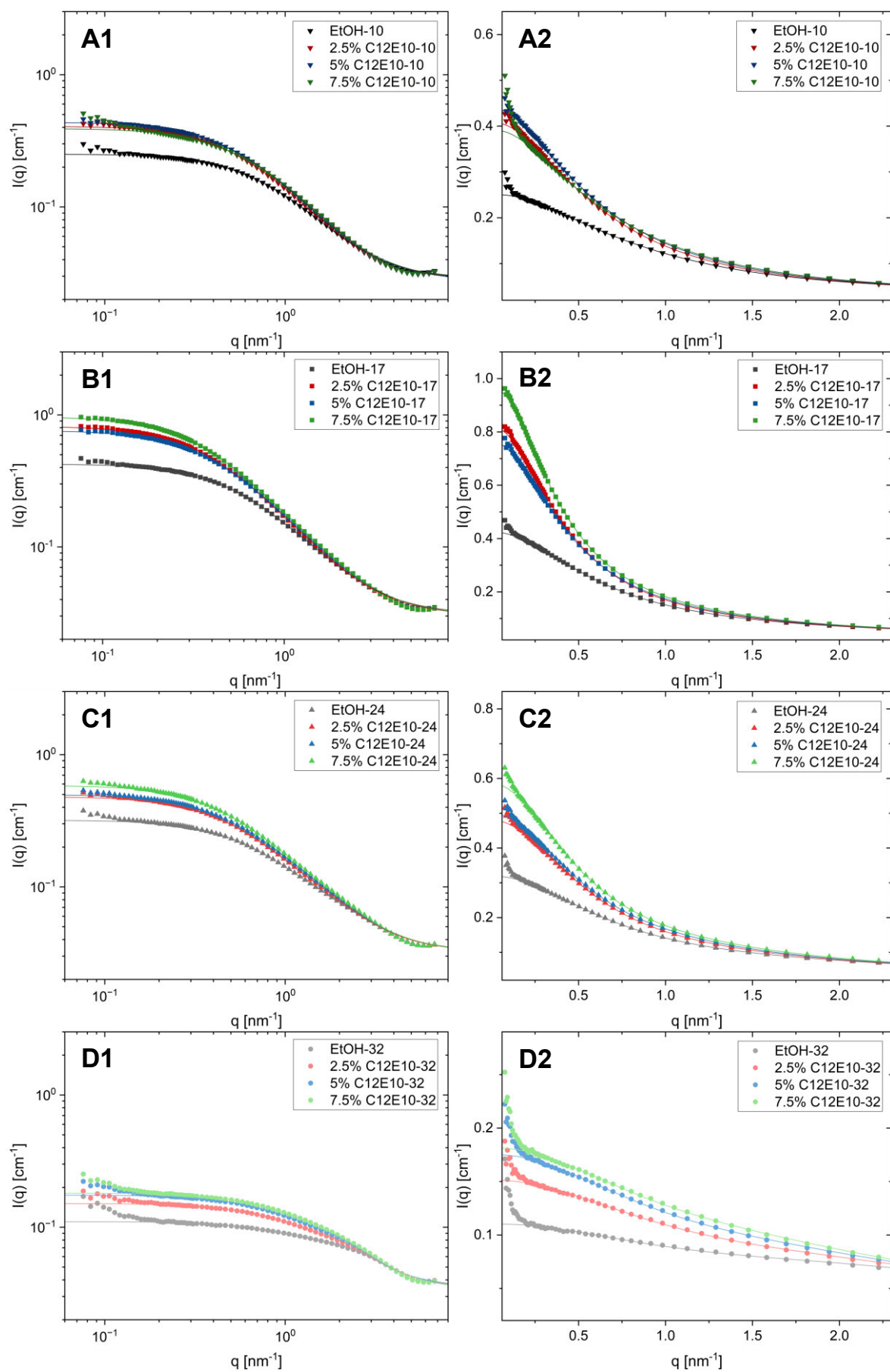


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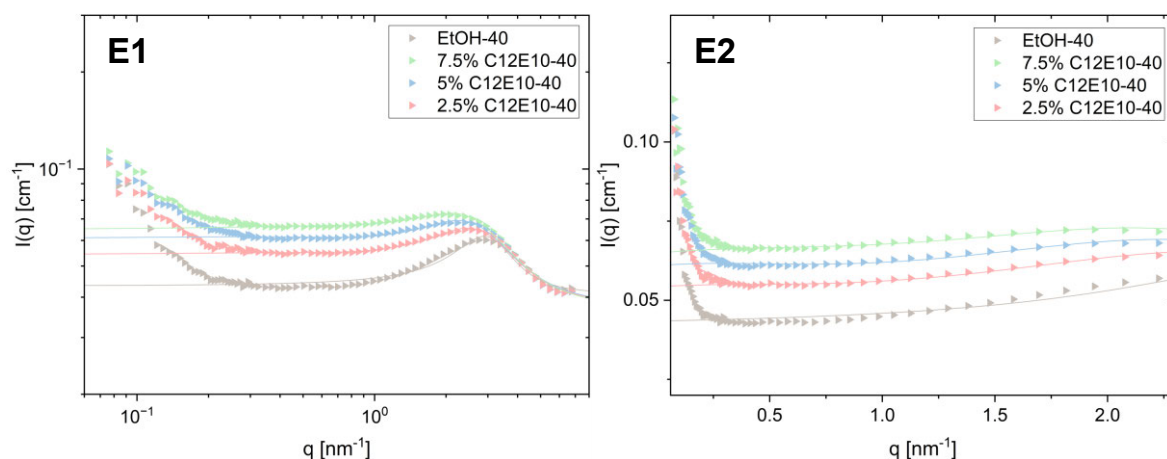


Figure 4.14: Scattering profiles of EtOH(+C12E10)/Oct/Water at distinct % Oct with increasing $c(\text{C12E10})$ in log-log scale (**A1-E1**) and lin-lin scale (**A2-E2**). Symbols are SAXS data and solid lines are OZ+BP-fitted curves. Model parameters are provided in Table SI 4 2.

For nonionic surfactants, no change in the mesostructure is observed at low % Oct. With 7.5% C12E10, a slight stabilization of the BIC structure is suggested, leading to its expansion to higher % Oct. Upon the addition of NaSal, an intermediate case is obtained: The pre-Ouzo region is expanded to 32% Oct, and the BIC phase is expanded to 40% Oct – no oil-rich phase is observed anymore. However, the form of the binodal is maintained upon the addition of NaSal and is shifted toward a more symmetric form upon the addition of ionic surfactant. The change in the mesostructure responsible for the increased solubilization of Oct in the water-rich region should be investigated in the following.

The individual contributions of OZ and BP to $I(q)$ and the peak positions obtained from the fits are shown in Figure 4.15 (**A1-G1** and **A2-G2**, respectively). The fit contributions are expressed as the $I_{BP}(q_p)$ intensity contribution to the total intensity $I(q)$, and they are compared based on the proportion of Oct in the sample. A value of 1 indicates no OZ contribution, while a value of 0 indicates no BP contribution. As discussed earlier, the fit model used involves only one contribution that produces peaks and captures only the most prominent one. In the following, the origin of BP contributions and parameters derived from them are marked as either arising from a peak at low- q (filled symbols) or from the Oct-OH cluster structure factor peak at high q (unfilled symbols).

The scattering patterns of pure SFME mainly show OZ behavior at 10-24% Oct. At 32 and 40% Oct, the BP contribution increases due to the emergence of the Oct-OH cluster structure factor peak around 2-3 nm^{-1} . Although pre-Ouzo aggregates can be well described by a single OZ contribution^{36,55} (see Figure SI 4 7), a minor BP contribution across the scattering profiles was also observed by Prévost *et al.*³⁷. They attributed the presence of the BP contribution to Oct-OH clusters inside pre-Ouzo aggregates³⁷. Lopian *et al.* and Prévost *et al.* explained the continuous decrease in q_p upon adding water and EtOH as an increase in the distance between Oct-OH clusters^{28,37}. The distances derived from q_p are later discussed (see Figure 4.17).

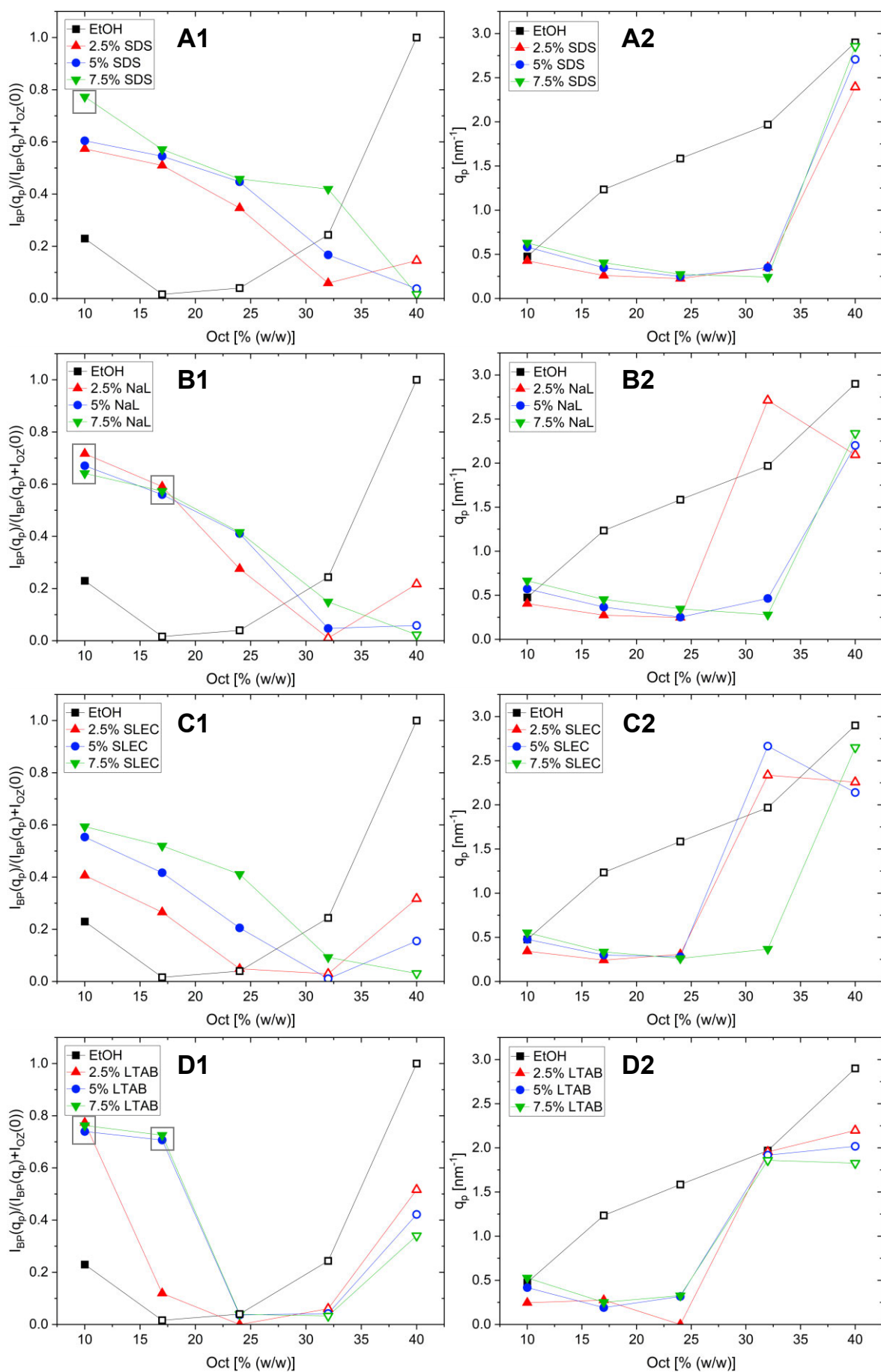


Figure continues.

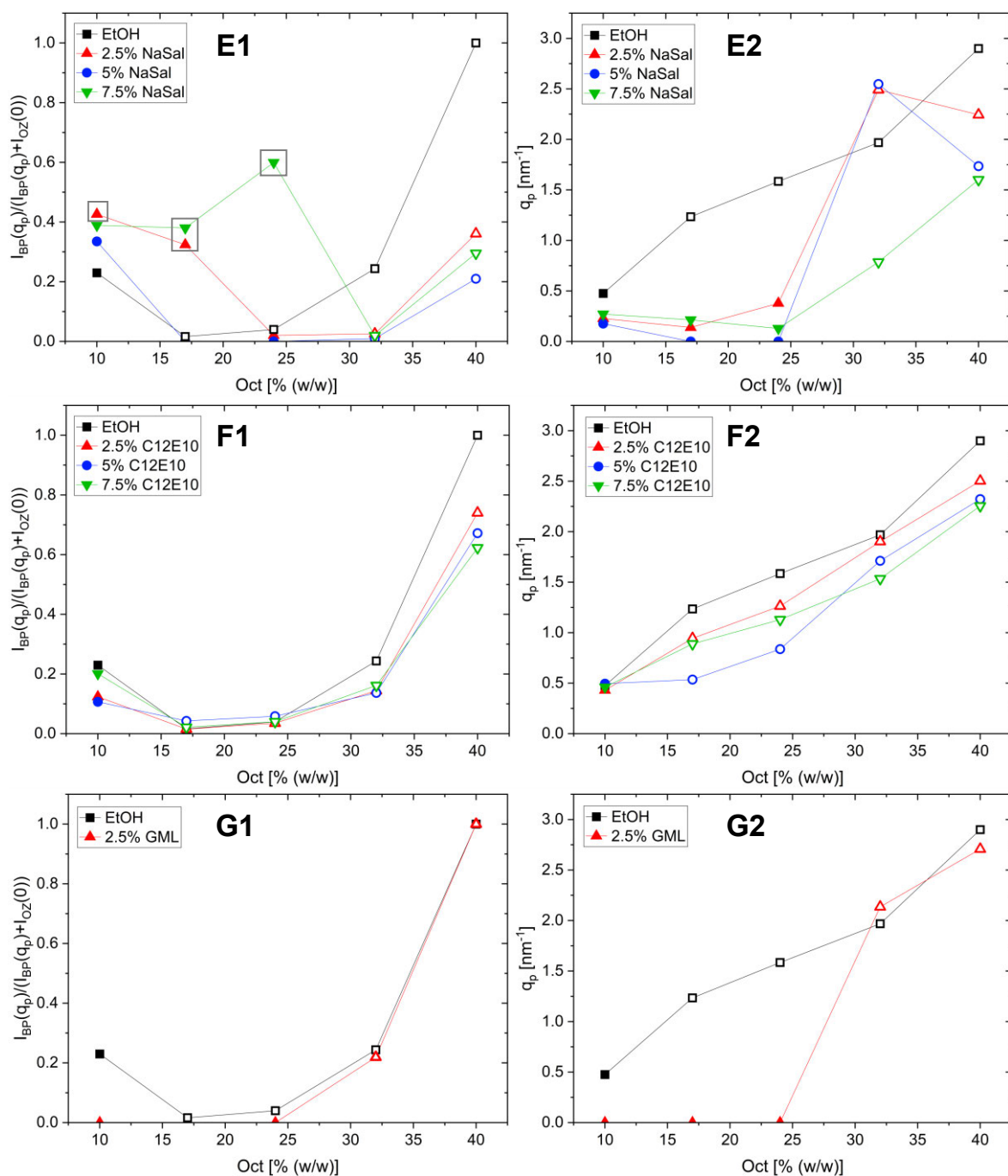


Figure 4.15: The $I_{BP}(q_p)$ intensity contribution to $I(q)$ (A1-G1) and the peak positions obtained from the fit (A2-G2) in pure EtOH/Oct/Water and with the addition of 2.5-7.5% compound SDS (A), NaL (B), SLEC (C), LTAB (D), NaSal (E), C12E10 (F), and GML (G). The fit contributions are presented as the ratio of $I_{BP}(q_p)$ to the sum of $I_{BP}(q_p)$ and $I_{OZ}(0)$ versus the proportion of Oct. A value of 1 signifies no OZ contribution, while a value of 0 signifies no BP contribution. Filled symbols assign the BP contribution to a peak at low q and unfilled symbols assign it to the peak produced from Oct-OH clusters at 2-4 nm⁻¹. Grey boxes indicate uncertain values. The uncertainty is provided in Table SI 4 3.

For the addition of SDS (A) and NaL (B), peaks linked to aggregate-aggregate spacing emerge in the water-rich region (see Figure 4.10 and Figure SI 4 3). The influence of this peak continues up to 32% Oct (except for 2.5% NaL). At 40% Oct, the influence of Oct-OH clusters dominates the BP contribution, as shown by the sharp increase in q_p to 2.1-2.9 nm⁻¹. Overall, the BP contribution decreases as % Oct increases, while the OZ contribution increases. Uncertain values are due to varying OZ contributions at low % Oct. This is illustrated in Figure SI 4 8. The uncertainties do not change the course of the

presented parameters and, therefore, are not further discussed. With 2.5% SDS/NaL, the Oct-OH cluster contribution is visible at 40% Oct. With 5% and 7.5% SDS/NaL, the Oct-OH cluster contribution is largely superimposed by OZ at 40% Oct. The scattering data for 2.5% and 5% SLEC (C) follow the same pattern as 2.5% SDS and NaL. At 7.5% SLEC, the scattering data are similar to those of 5% and 7.5% SDS/NaL. The addition of LTAB (D) creates a U-shaped distribution of OZ and BP contributions in Figure 4.15, with BP (arising from a peak at low q) dominating at low Oct proportions and reappearing at 40% Oct (arising from the Oct-OH cluster structure factor peak), while OZ dominates at intermediate Oct levels. The Oct-OH cluster peak primarily affects BP contribution at 32% and 40% Oct, not just at 40% Oct as with SDS, NaL, and partly SLEC. The peak position is shifted to $q = 1.8\text{--}2.2\text{ nm}^{-1}$. For the addition of NaSal (E), it is difficult to identify a clear trend in the contributions of OZ and BP. At 10% and 17% Oct, the contribution magnitude flips depending on the fitted data range (see Table SI 4 3). Samples with 5% NaSal were measured too close to the binodal. At 32% Oct, the OZ contribution clearly dominates. At 40% Oct, the BP contribution becomes more dominant. At both points, peaks are assigned to Oct-OH clusters. The peak positions are similar to those observed with SLEC. The addition of C12E10 (F) does not significantly alter the proportion of fit contributions compared to pure SFME. At all concentrations, the scattering data mainly follow OZ behavior at 10–24% Oct. At 32% and 40% Oct, the BP contribution increases due to a more pronounced presence of Oct-OH clusters. Scattering data of 2.5% GML at 10–24% Oct are fitted by OZ only. BP contributions at 32% and 40% Oct are assigned to the Oct-OH cluster peak and are similar to pure SFME and C12E10. The contribution of Oct-OH clusters remains visible and influences $I(q)$ at lower $c(\text{ionic additive})$, but it is not modeled by the fit at high $c(\text{ionic additive})$. Therefore, it is not possible to determine whether the structure of Oct-OH clusters changes upon the addition of anionic surfactants. To do so, the fit model would need to be adjusted to include an additional non-zero Lorentzian function. Going from 10% Oct to 40% Oct, three different behaviors of q_p are observed. These borderline cases are shown schematically in Figure 4.16.

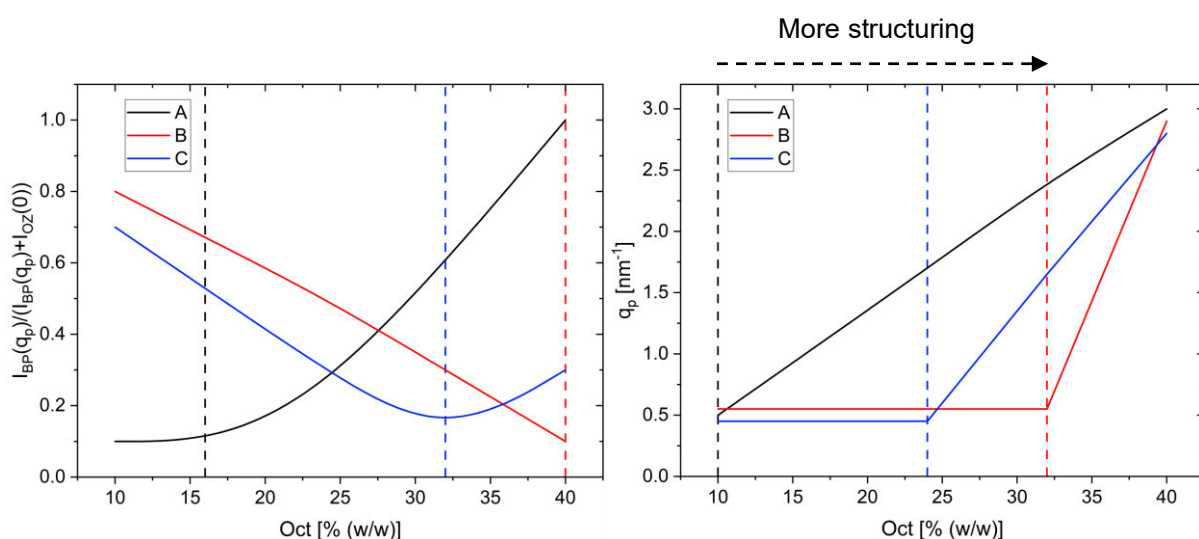


Figure 4.16: Borderline cases A, B, and C of the course of the $I_{BP}(q_p)$ intensity contribution (left) and q_p (right).

- A: The $I_{BP}(q_p)$ intensity contribution increases with increasing % Oct. q_p increases gradually and is assigned to Oct-OH clusters from 17% Oct onwards. This is the case for pure SFME and upon adding C12E10.
- B: The $I_{BP}(q_p)$ intensity contribution decreases with increasing % Oct. q_p is low at 10-32% Oct, and sharply increases at 40% Oct. In contrast to the pure SFME, only q_p at 40% Oct is assigned to Oct-OH clusters. This is the case for SDS, 7.5% NaL, and 7.5% SLEC.
- C: The $I_{BP}(q_p)$ intensity contribution decreases up to 32% Oct and increases at 40% Oct. q_p is low at 10-24% Oct, and sharply increases at 32-40% Oct. q_p is assigned to Oct-OH clusters at 32 and 40% Oct. This is the case for LTAB, NaSal, 2.5% NaL, and 2.5-5% SLEC.

Case C represents an intermediate case between A and B. The transition from a predominant Oct droplet peak at low q to a predominant Oct-OH cluster peak at high q shifts toward higher % Oct when ionic additives are present (marked with dashed lines). Together with β obtained from DLS (see Figure 4.7) and scattering patterns (see Figure 4.9, Figure 4.10, and Figure SI 4 3-6), this shift suggests increased structuring and stabilization of pre-Ouzo aggregates and thus destabilization of BIC. The fact that no structure factor peaks emerge upon adding NaSal suggests that the nature of pre-Ouzo aggregates might be more closely related to that of the pure SFME and C12E10 than with the addition of ionic surfactants.

In Figure 4.17, the interaggregate distances or mesh sizes d are presented. The values were calculated from q_p using equation 4.5. The color map displays the $I_{BP}(q_p)$ intensity contribution to indicate the reliability of d , a parameter derived from the BP fit contribution.

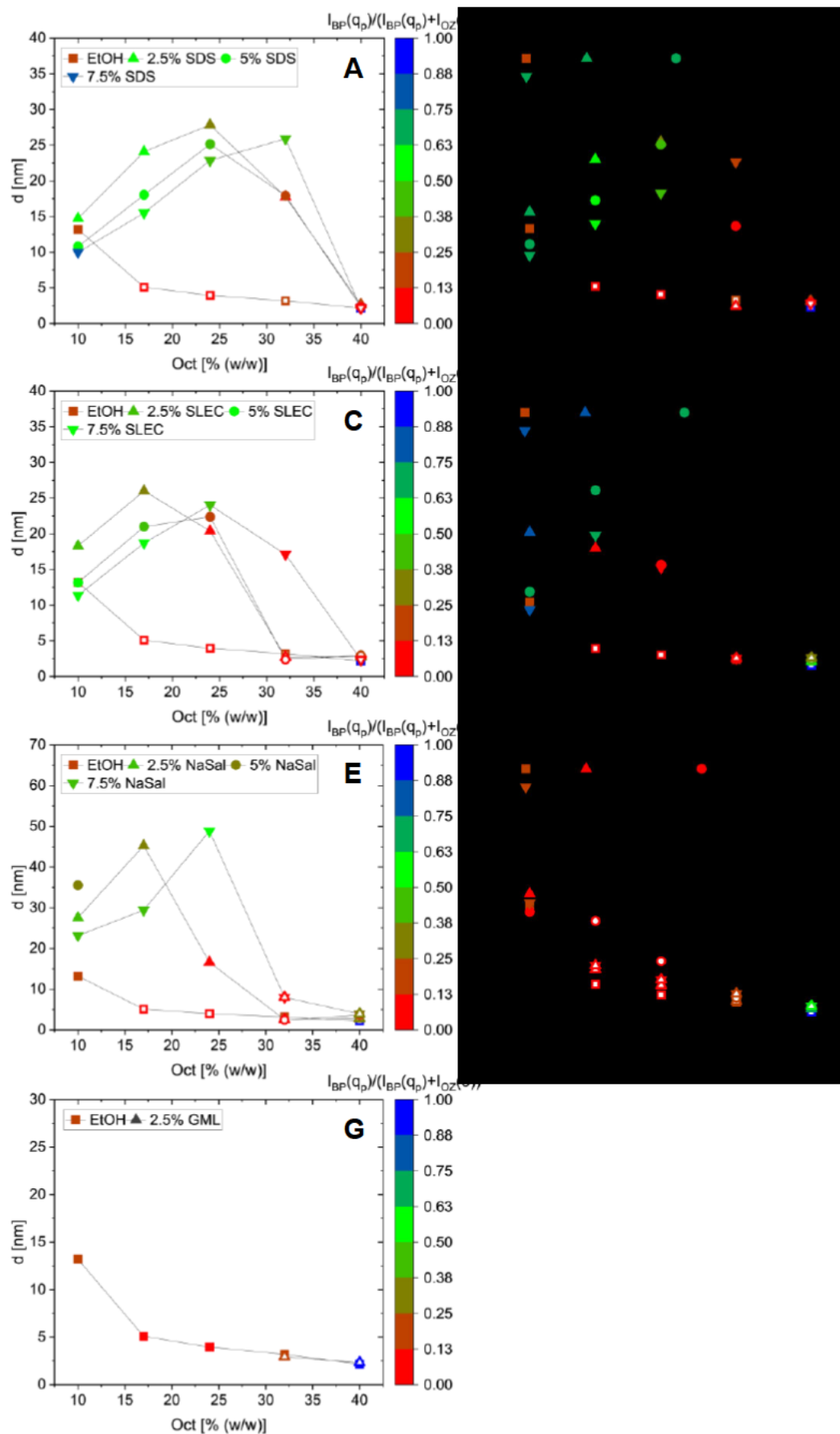


Figure 4.17: Interaggregate distances d calculated from q_p for the pure SFME EtOH/Oct/Water and with the addition of 2.5-7.5% compound SDS (A), NaI (B), SLEC (C), LTAB (D), NaSal (E), C12E10 (F), and GML (G). Filled symbols assign the BP contribution to a peak at low q , and unfilled symbols assign it to the peak produced from Oct-OH clusters. The reliability of the values is indicated by the $I_{BP}(q_p)$ intensity contribution.

The course of parameter d is complementary to q_p , based on its calculation. Considering the $I_{BP}(q_p)$ intensity contribution, reliable values are obtained for 10-24% Oct with SDS (**A**) and NaL (**B**) at all concentrations, with SLEC (**C**) and LTAB (**D**) at 5% and 7.5%, and at 40% Oct for the pure SFME. In the latter case, $d = 2.2$ nm marks the distance between Oct-OH clusters in Oct^{11,28}. Interaggregate distances behave similarly upon addition of nonionic surfactants (**F** and **G**, as far as evaluable regarding the low $I_{BP}(q_p)$ intensity contribution).

With the addition of ionic surfactants (**A-D**), interaggregate distances $d = 10-15$ nm are comparable to the pure SFME or even slightly smaller and increase at intermediate % Oct to $d = 20-35$ nm. Approaching 40% Oct, values drop again to values comparable to the pure SFME. In the case of pure SDS micelles^{118,119}, the intermicellar distance would be $d = 2\pi/1.55 \text{ nm}^{-1} = 4.05$ nm (as discussed with q_p in Figure 4.15). Blahnik observed interaggregate distances of 5-16 nm and suggested the progressive formation of mixed micelles⁹⁰. Marcus *et al.* observed the formation of smaller aggregates than in the pure SFME and assigned it to increased repulsion due to the formation of mixed micelles⁵⁵. When increasing the Oct proportion, it can be assumed that Oct does not dissolve in the solvent at the molecular level but instead participates in structure formation. If the aggregate concentration increases, d decreases. If the aggregate concentration stays the same and existing aggregates are enriched with Oct, d remains constant. The only way to increase d values is by decreasing the aggregate concentration while simultaneously increasing the aggregate size. Such aggregates exhibit a lower surface charge, which explains the disappearance of the structure factor peak as indicated by SAXS profiles in Figure 4.10.

With the addition of NaSal (**E**), interaggregate distances $d = 23-28$ nm are larger than the pure SFME and increase to $d = 45-49$ nm at 17-24% Oct. d drops to values comparable to the pure SFME at 40% Oct. Together with increasing forward scattering, the formation of fewer and larger aggregates is suggested. Compared to aggregates formed upon the addition of ionic surfactants, aggregates are suggested to be larger with the addition of NaSal.

To analyze the evolution of aggregate size more closely, the correlation lengths ξ_{OZ} and ξ_{BP} are shown in Figure 4.18. When the scattering data are modeled solely by OZ or BP contributions, the corresponding other ξ values are zero. The color map displays the $I_{BP}(q_p)$ intensity contribution to indicate the reliability of ξ_{OZ} and ξ_{BP} , as they are derived from different fit contributions.

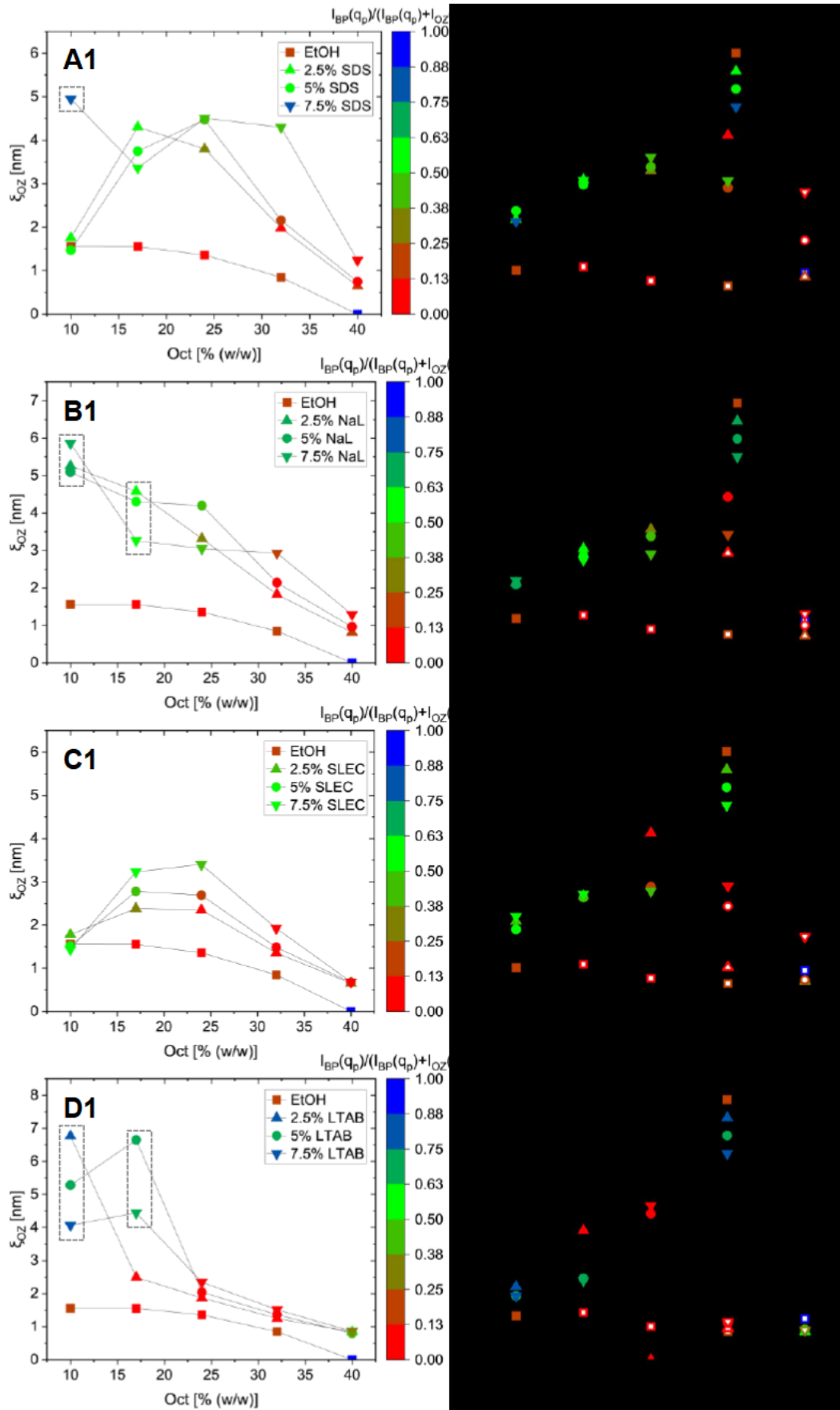


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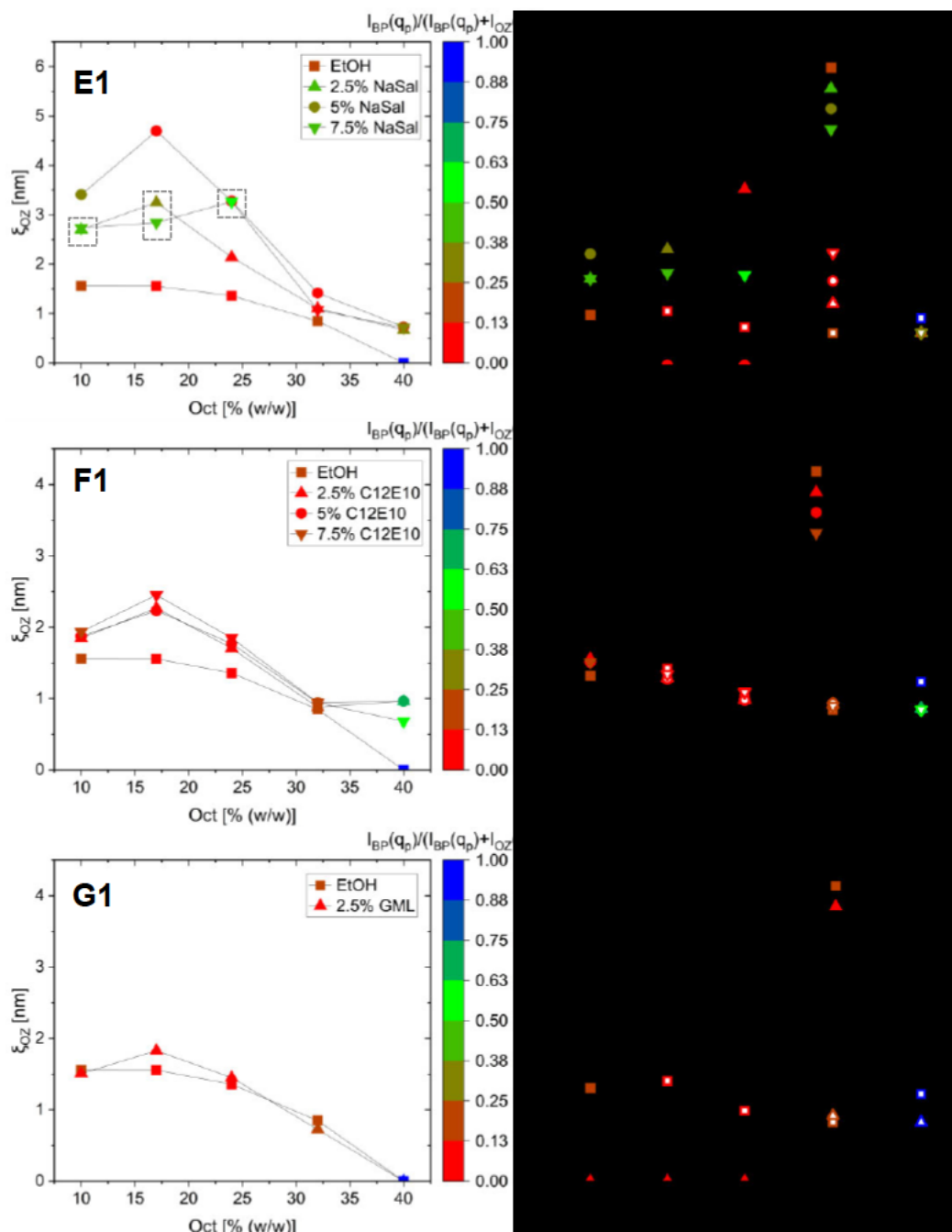


Figure 4.18: The correlation lengths ξ_{OZ} and ξ_{BP} obtained from the combined OZ+BP fit model for pure EtOH/Oct/Water and with the addition of 2.5-7.5% compound SDS (A), NaL (B), SLEC (C), LTAB (D), NaSal (E), C12E10 (F), and GML (G). Filled symbols assign the BP contribution to a peak at low q and unfilled symbols assign it to the peak produced from Oct-OH clusters. Grey boxes indicate uncertain values. The uncertainty is provided in Table SI 4 3. The reliability of the values is indicated by the $I_{BP}(q_p)$ intensity contribution.

At 10-24% Oct, the scattering data of the pure SFME are dominated by the OZ contribution, making the values of ξ_{BP} less reliable³⁷. The derived correlation length ξ_{OZ} is highest at 10-17% Oct, with $\xi_{OZ} = 1.56$ nm, and decreases at higher Oct levels. This value is slightly smaller than pre-Ouzo aggregate values reported in the literature³⁷. At 32% Oct, the scattering data are described by a combination of a dominant OZ contribution and a minor BP contribution with $\xi_{OZ} = 0.85$ nm and $\xi_{BP} = 0.64$ nm. At 40% Oct, the scattering is dominated by Oct-OH clusters, yielding $\xi_{BP} = 0.95$ nm.

The pattern remains consistent with the addition of C12E10 (**F**) and GML (**G**) at 10-32% Oct, indicating that the mesostructure remains essentially unchanged, but aggregation slightly increases in the pre-Ouzo region, leading to $\xi_{\text{OZ}} = 1.5\text{-}2.3$ nm. At 40% Oct with 7.5% C12E10, the scattering pattern (see Figure 4.14), $\xi_{\text{OZ}} = \xi_{\text{BP}} = 0.6\text{-}0.7$, and $I_{\text{BP}}(q_p)/(I_{\text{BP}}(q_p)+I_{\text{OZ}}(0)) \approx 0.5$ indicate the presence of the BIC phase^{28,37}. In contrast to the pure SFME and C12E10, scattering data with 2.5% GML at 10-24% Oct are best fitted by pure OZ. Upon adding SDS (**A**), the ξ_{BP} values at 10-32% correspond to the peak at low q . While at 10-24% Oct (and at 32% Oct with 7.5% SDS), both fit contributions are generally equally present, OZ dominates at 32% and 40% Oct (and at 24% Oct with 2.5% SDS). The point at 10% Oct with 7.5% SDS is an exception: The OZ contribution varies with the selection of fitted data points, and β_{OZ} decreases as the fitting range narrows (see Table SI 4 3). Additionally, the BP contribution is dominating. Thus, little attention is given to the comparably high ξ_{OZ} value. The ξ_{OZ} and ξ_{BP} values generally increase from 10% to 24% Oct and decrease at 32-40% Oct. The maximum values are $\xi_{\text{OZ}} = 4.3$ nm at 17% Oct with 2.5% SDS and $\xi_{\text{OZ}} = 4.6$ nm at 24% Oct with 5% and 7.5% SDS. For ξ_{BP} , the maximum is between 3.4 nm and 3.6 nm at 24% Oct across all SDS concentrations. The pattern appears different when NaL is added to the system (**B**) rather than SDS. ξ_{OZ} values seem to decrease with increasing % Oct. Since the values at 10% and 17% Oct are highly uncertain and decrease as the fitting range narrows (see Table SI 4 3), the values should be evaluated with caution. ξ_{BP} values are more reliable as the contribution of BP to the total intensity dominates in that area. ξ_{BP} values are between 1.8-1.9 nm at 10% Oct. Therefore, the behavior with the addition of NaL could also be similar to that observed with SDS. The pattern for 2.5% to 7.5% SLEC (**C**) is similar to that observed with SDS, with ξ_{OZ} and ξ_{BP} values slightly lower than those obtained with SDS. Upon adding LTAB (**D**), the pattern of ξ_{OZ} and ξ_{BP} values is less forward. ξ_{OZ} values are high and uncertain at 10 and 17% Oct and behave similar to those with NaL upon narrowing the fitting range (see Table SI 4 3). The corresponding ξ_{BP} values range from 1.5 nm to 3.0 nm with increasing % Oct. Both ξ values decrease similarly at 32-40% Oct. The evaluation of EtOH+NaSal/Oct/Water (**E**) is also less straightforward because samples with 5% NaSal were measured too close to the binodal, and several data points with 2.5% and 7.5% NaSal are uncertain. In contrast to the uncertain values obtained for ionic surfactants, the trend with NaSal goes toward higher ξ_{OZ} values than shown (see Table SI 4 3). At 32-40% Oct, the OZ contribution dominates, and ξ_{OZ} values are slightly higher than in pure SFME.

Summarizing the ξ_{OZ} and ξ_{BP} values obtained from the fit upon adding nonionic and ionic compounds to the pure SFME, four different behaviours can be distinguished. They are schematically shown in Figure 4.19.

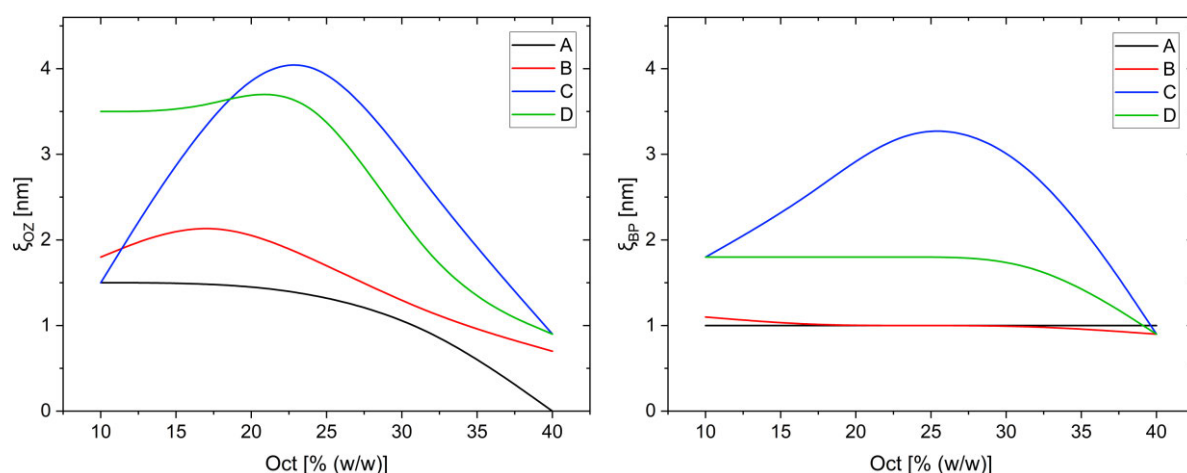


Figure 4.19: Schematic behaviors A-D of ξ_{OZ} and ξ_{BP} . The height of the displayed maxima depends on the additive and its concentration.

- A: ξ_{OZ} remains constant between 10% and 24% Oct and decreases for 32% and 40% Oct. ξ_{BP} remains constant across the investigated amount of Oct. This is the case for the pure SFME.
- B: ξ_{OZ} is slightly increased compared to A and forms a shallow maximum at intermediate % Oct. ξ_{BP} remains nearly constant across the investigated amount of Oct, with slightly higher values at low % Oct and slightly lower values at high % Oct. This is the case when C12E10 is added.
- C: ξ_{OZ} strongly increases up to a maximum at higher % Oct compared to case B and subsequently decreases with increasing % Oct. ξ_{BP} behaves similarly. This is the case for the addition of ionic surfactants. The height of the maximum and its position at % Oct depends on the surfactant and its concentration.
- D: The course of ξ_{OZ} describes a plateau at comparably high values, forms a shallow maximum at intermediate % Oct, and then decreases. The course of ξ_{BP} is similar, with values between those of cases A/B and C, without forming a maximum. This is the case for the addition of NaSal, but it should be noted that data with 5% NaSal are not included.

The schematic courses of the correlation lengths indicate an increase in the aggregate size of the pre-Ouzo structures upon the addition of all analyzed compounds. The increase is more pronounced for ionic additives than for nonionic ones. At 40% Oct, the formation of oil-rich aggregates is suppressed in favor of an enlarged pre-Ouzo or BIC phase. The nature of the enlarged aggregates is discussed in the next chapter.

4.4.2.3. Qualitative Evaluation of the Distribution of EtOH Between Pseudo-Phases Using a Modified Teubner-Strey Fit Model

Samples of 10-32% Oct were also fitted to a modified Teubner-Strey (TS) model. 40% Oct was not fitted, as the scattering data in most cases did not fit at all. The same was true for 2.5% GML. The fits are shown in Figure SI 4 9. The model fits the SAXS data well at low q , but does not capture the pattern at high q due to a q^{-4} decay in the model and a smaller decay in the SAXS data. The fact that the scattering data cannot be fitted by TS shows that, regardless of surfactant concentration or position in the phase diagram, these are not classical microemulsions^{11,37}. The fitting range was narrowed to the data well described by the model. The parameter $x_E(A)$, i.e., the proportion of EtOH in the polar EtOH-water pseudo-phase, is thus only evaluated qualitatively. All fit parameters are given in Table SI 4 4.

$x_E(A)$ generally decreases with increasing % Oct in the pure SFME and across all additive concentrations. In the case of pure SFME and with the addition of C12E10, $x_E(A)$ is largest at 10% Oct with $x_E(A) \approx 0.6$, and lowest with $x_E(A) \approx 0.1$ at 32% Oct. When adding ionic additives, the trend remains, but $x_E(A)$ values are generally larger, with $x_E(A) \approx 0.8$ at 10% Oct and $x_E(A) \approx 0.3$ at 32% Oct. It is important to emphasize that the use of the TS model implies the presence of two pseudo-phases separated by a sharp interface. Thus, changes in interfacial composition are not accounted for by that model. One hypothesis for the values obtained for $x_E(A)$ is as follows: a decrease in the binodal at a given Oct concentration indicates an increase in water and a decrease in EtOH when keeping the distance to the binodal fixed. This is only possible with the addition of ionic surface-active compounds, as observed in the phase diagrams in Figure 4.5. Assuming that these compounds are more or less located in the interface, EtOH might be salted out from the interface into the EtOH-water pseudo-phase due to stabilization of the mesostructures and reduction of the fluctuating interface. For a more detailed analysis of solvent distribution, small-angle neutron scattering (SANS) measurements and determination of tie lines would be required.

Marcus *et al.*⁵⁵ and Schöttl *et al.*⁵⁹ reported salting-out of EtOH from the aqueous pseudo-phase into the interface and into aggregates upon addition of classical salts, resulting in increased aggregate size and shifting the sample closer to demixing. Marcus *et al.* reported the opposite effect upon the addition of antagonistic salts and SDS: as in the present examples, $c(\text{EtOH})$ increased in the aqueous pseudo-phase, with EtOH present in the interface and stabilization of present aggregates or favouring the formation of smaller micelle-like aggregates. In the case of SDS, the interface is proposed to include more Oct as $c(\text{SDS})$ increases⁵⁵. Lopian *et al.* observe a decrease in the biphasic region and a stabilization of pre-Ouzo aggregates via increased repulsion with the addition of 2-4 M sulfuric acid. They explain the increased repulsion with an accumulation of EtOH at the interface or a secondary hydration force arising from the electrolyte present²⁸.

The parameters evaluated in the previous sections are combined with results from the literature on the distribution of solvents and summarized into a mesoscale picture of the processes that occur upon adding very small additive concentrations.

4.4.3. Summary and Schematic Representation of the Mesostructure

Taking all evaluations into account, the following impact of the analyzed additives is suggested. The resulting changes in the mesostructure of EtOH/Oct/Water are schematically illustrated in Figure 4.20.

Decreasing d values (see Figure 4.17) and first slightly increasing forward scattering $I(q \rightarrow 0)$ from SAXS (see Figure 4.7) indicate the formation of additional Oct-rich aggregates with increasing % Oct in the pre-Ouzo region of the pure SFME before transitioning to the BIC phase. A slight change in the aggregate size with increasing % Oct could also be possible.

Adding C12E10 and GML does not significantly alter the binodal of EtOH/Oct/Water (see Figure 4.5). The apparent coherence factors β obtained from DLS and forward scattering $I(q \rightarrow 0)$ (see Figure 4.7) are generally larger than those of pure SFME but behave similarly. No peaks appear in the SAXS profiles at any concentration, and the scattering patterns resemble classical OZ behavior (see Figure 4.13 and Figure 4.14). However, the BP contribution is present at all points on the binodal (except for GML) and increases with increasing % Oct. While at 10% Oct, BP is assigned to a peak in low q , it shifts to higher q with increasing % Oct, indicating the growing contribution of an Oct bulk phase (see Figure 4.15). The distance between scatterer centers d , calculated from q_p , decreases from 12-15 nm to about 3 nm as % Oct increases in the pure SFME and upon addition of non-ionic surfactants (see Figure 4.17). While correlation lengths ξ_{OZ} are slightly larger than those of the pure SFME in the pre-Ouzo region, correlation lengths ξ_{BP} remain unchanged (see Figure 4.18). At 40% Oct, the BIC phase is stabilized, and the formation of reverse aggregates is suppressed, as indicated by the scattering pattern and $\xi_{OZ} \approx \xi_{BP}$. Overall, these results suggest that adding nonionic surfactants has a minor impact on the mesostructure of EtOH/Oct/Water in the pre-Ouzo region. Slightly larger ξ_{OZ} values, together with higher values of β and $I(q \rightarrow 0)$, suggest a slight increase in pre-Ouzo aggregate size. The binodal in that area is reduced only slightly. The solubility of C12E10 is high in EtOH and it is also soluble in Oct, which is why reduced interfacial activity can be assumed. However, the binodal is reduced somewhat and the BIC phase at 40% Oct with 7.5% C12E10 is stabilized, so a residual affinity for the interface remains. The potential nature of these aggregates or changes in their interfaces cannot be derived from the presented experiments.

The binodal is reduced gradually with increasing NaSal concentration. The slightly asymmetrical shape of the binodal remains unchanged (see Figure 4.5). β and $I(q \rightarrow 0)$ (see Figure 4.7) are generally larger

than those of pure SFME but behave similarly. Like with nonionic surfactants, no peaks appear in the SAXS profiles, and the scattering patterns resemble classical OZ behavior (see Figure 4.11). The BP contribution is more pronounced than that of nonionic surfactants and the pure SFME at lower % Oct and less pronounced at 40% Oct. BP contributions at 10-17% Oct (and 24% Oct at 7.5% NaSal) are assigned to structure factor peaks in low q , and BP contributions at 32-40% Oct are assigned to structure factor peaks in high q (see Figure 4.15). This suggests increased structuring and a stabilization of existing pre-Ouzo aggregates with increasing $c(\text{NaSal})$. The interscatterer distance first increases from 23-28 nm to about 45-49 nm as % Oct increases and then decreases to values comparable to the pure SFME (see Figure 4.17). Both correlation lengths are larger than those from the pure SFME and nonionic surfactants and peak at intermediate % Oct (see Figure 4.18). The form of the scattering data at 40% Oct suggests stabilization of the BIC phase. NaSal is an ionic hydrotrope, which implies its affinity for surfaces. However, its solubility in EtOH and water is high, and its affinity for the diffuse interface might be reduced (as with nonionic surfactants).

In the literature, increased pre-ouzo aggregates upon the addition of classical salts were assigned to salting-out of EtOH from the aqueous pseudo-phase and a resulting demixing of the composition⁵⁹. This is clearly not the case here, as the binodal decreased with the addition of NaSal. Going to antagonistic salts, a surfactant-like behavior in the interface was observed, leading to its charging and stabilization^{55-58,122,123}. With sufficiently high salt concentrations, micelle-like structures were observed⁵⁵. This was also observed for NaSal at high concentrations¹²⁰. Based on NaSal being an ionic hydrotrope, the concentrations used in the present case, and the absence of peaks in the scattering patterns, minimal charging of the interface stabilizing existing mesostructures and resulting in the formation of larger pre-Ouzo aggregates is proposed.

When ionic surfactants are added, the binodal also decreases gradually with increasing surfactant concentration, outperforming the effect of NaSal. The slightly asymmetrical shape of the binodal becomes more symmetrical with a comparably larger reduction at the Oct-poor side (see Figure 4.5). β and $I(q \rightarrow 0)$ (see Figure 4.7) are larger than those of the pure SFME and form maxima at higher % Oct with increasing $c(\text{surfactant})$. The SAXS profiles show peaks and shoulders at 10-17% Oct. Both structural motifs become more pronounced with increasing surfactant concentration and gradually disappear when shifting to higher % Oct (see Figure 4.13, Figure 4.14, and Figure SI 4 3-6). The power law increased to q^{-2} or q^{-3} but never reached the Porod limit q^{-4} . The BP contribution is most pronounced at 10% Oct and decreases gradually toward 32% Oct at 2.5% and 5% ionic surfactant, and toward 40% Oct to 7.5% ionic surfactant. Complementary to the pure SFME, peaks at 10-24/32% Oct are assigned to the peak in low q , indicating increased structuring. At 32-40% Oct, the BP contribution shifts to the peak in high q (see Figure 4.15). The behavior is less pronounced when the cationic surfactant LTAB is added. The BP contribution is most pronounced at 10% Oct and decreases sharply

to 24% Oct before increasing again. Peaks at 32% and 40% Oct are assigned to the peak in high q . The behavior of the interscatterer distance follows the course of the BP contributions complementarily. As BP decreases, d increases from values comparable to the pure SFME or slightly smaller to 21-28 nm and decreases again to values similar to the pure SFME (see Figure 4.17). ξ_{BP} values for all anionic surfactants are larger than those of the pure SFME and generally increase with increasing % Oct. At 40% Oct, they decrease or drop to values similar to the pure SFME. Similar to d , ξ_{BP} values of LTAB peak at 17-24% Oct and decrease at lower or higher % Oct. ξ_{OZ} values at 10% Oct with SDS 7.5% and at 10-17% Oct with all NaL and LTAB concentrations are highly uncertain. Taking the uncertainty into account, a reverse U-shaped course of ξ_{OZ} values is suggested, with values similar to or smaller than those for the pure SFME at low % Oct, increasing strongly with increasing % Oct, and decreasing again to values larger than with the pure SFME (see Figure 4.18). Overall, these results indicate a change in the mesostructure of EtOH/Oct/Water. At low % Oct, the significant reduction of the binodal, the structure factor peak formation in SAXS profiles, together with increased $I(q \rightarrow 0)$ and decreased interscatterer distances, indicate the formation of a higher concentration of smaller pre-Ouzo aggregates compared to the pure SFME. Anionic surfactants in the interface induce charging and structuring due to their low solubility in EtOH. With increasing % Oct, the aggregates become larger than those in the pure SFME and less defined than those at 10% Oct, as indicated by the loss of the structure factor peak in the SAXS profile, and by increasing interscatterer distances and correlation lengths. The presence of a comparably small amount of ionic surfactant significantly stabilizes pre-Ouzo aggregates, extending this region to higher % Oct.

Compared to the observations of Marcus *et al.*⁵⁵, it can be concluded that the addition of small amounts of ionic surfactants is sufficient to produce smaller aggregates in the Oct-poor region. Unlike with the addition of comparably high concentrations of SDS in the case of Marcus *et al.*, Oct droplets stabilized by surfactants instead of mixed micelles are formed at low $c(\text{SDS})$ (or ionic surfactant in general). The transition between these states is gradual. Compared to the addition of NaSal, the surface activity of classical ionic surfactants is higher, thus, inducing a proportionally higher charging than with the addition of NaSal. Therefore, at 10% Oct, larger aggregates are formed with NaSal, and smaller ones are formed with anionic surfactant.

The critical point (CP) in a ternary system marks equal presence of both pseudo-phases and fluctuations of molecular aggregate concentration³⁷. Summarizing the results discussed, a shift of the critical point toward higher % Oct is proposed upon the addition of anionic surfactants: the OZ contribution increases with increasing % Oct before a prevailing BP contribution due to Oct-OH clusters dominating at high q (see Figure 4.15 and Figure 4.16). The course of the correlation lengths forms a maximum at 24-32% Oct compared to at 17% Oct of pure SFME and with the addition of nonionic surfactants (see Figure 4.18). Following the argumentation of Prévost *et al.*, these parameters indicate

a shift of CP toward increased % Oct. Further indications in this context are a shift in the maximum observed for β and $I(q \rightarrow 0)$ (see Figure 4.7). A shift of CP to 24% Oct could also be indicated for the addition of NaSal, but parameters are less straightforward.

LTAB generally showed less pronounced structuring compared to anionic surfactants. As discussed before, LTAB has an ammonium headgroup, which, from a Hofmeister perspective, is considered to be rather soft and weakly hydrated. The carboxylate headgroup present in NaL and SLEC is considered hard and strongly hydrated. The sulfate headgroup of SDS is considered rather soft^{94–96}. Given the scattering patterns in the Oct-poor region, it can be suggested that the peak formation is strongest with NaL, as the carboxylate group is strongly hydrated and thus induces the most pronounced structuring. LTAB consequently induces less structuring.

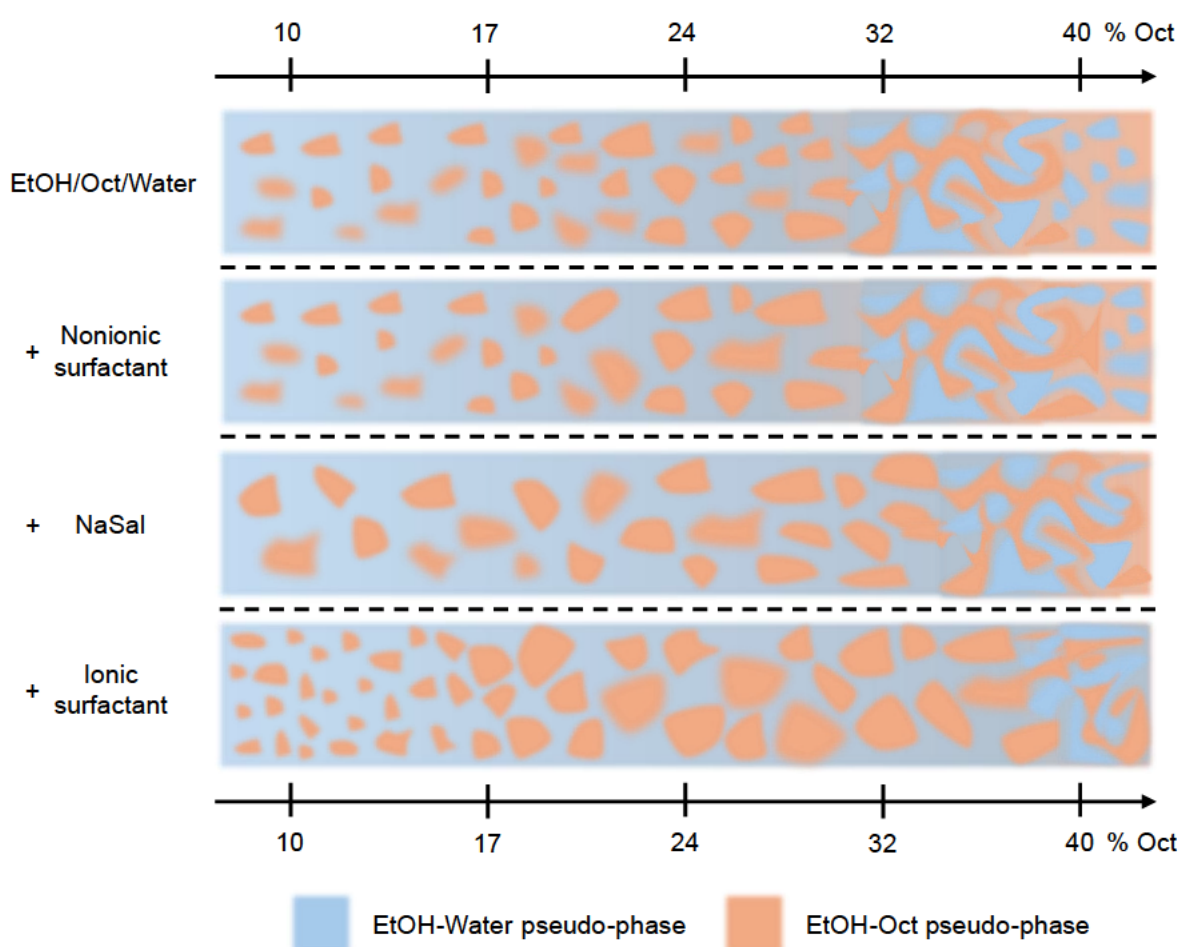


Figure 4.20: Schematic illustration of the effect on the mesostructure of EtOH/Oct/Water with the addition of nonionic surfactant, ionic hydrotrope, and ionic surfactant at a concentration of 2.5-7.5% in proportion of EtOH.

In summary, it is proposed that the impact of additives on the phase boundary and mesostructures can be estimated by

- the presence or absence of a charge
- their solubility in the SFME solvent compounds
- and, in the case of an ionic surfactant, the hydration of the headgroup.

The presented study extends the study of Marcus *et al.*⁵⁵ toward smaller additive concentrations, investigation of different surface-active compounds, and investigation of the former biphasic region. It systematically follows on work by Jonas Blahnik⁹⁰ and could verify some of his proposed hypotheses:

- the combination of surface-active and ionic character is crucial to achieve maximum reduction of the binodal with minimal additive concentration
- the headgroup of the ionic surfactant influences its performance in reducing the biphasic region
- SDS is located in the interface of the mesostructures and acts similar to an antagonistic salt by charging the interface

Going from a fundamental structure-related perspective to an application-related one, the findings connect the areas of SFME- and μ E-based applications: as mentioned in the introduction, μ Es have a wide range of applications, but their use in food or health care is limited due to their high surfactant content. SFMEs tackle that problem, but their polydisperse, fluctuating structures can provide a major difference and/or disadvantage to the relatively monodisperse compartmentalization of μ Es. By adding just a small amount of surfactants, an intermediate state between these two systems is achieved, facilitating a reduction in surfactant content while simultaneously stabilizing mesostructures. The reduction of the binodal is an interesting phenomenon when reducing organic solvents and increasing the water content in a formulation. This is, for example, the case in the perfume industry, where EtOH may be replaced and the water content increased. Due to the insolubility of most fragrance compounds in water, and the restriction of surfactant use in cosmetics^{72,125–127}, a solution in that direction is possible.

4.5. Conclusion and Outlook

The influence of additives on the mesostructure of SFMEs has rarely been studied in the literature. For a better understanding of the structure and their behavior in multicomponent mixtures in industrial applications, grasping these mesoscale influences is essential. The present study focused on the system EtOH/Oct/Water and three different classes of surface-active compounds as additives: ionic surfactants, nonionic surfactants, and an ionic hydrotrope. Their impact on the liquid-liquid phase boundary at concentrations between 2.5% to 7.5% in weight percent of EtOH was examined through phase diagrams, and the mesostructure was investigated using DLS and SAXS measurements. Nonionic surfactants C12E10 and GML did not significantly affect the binodal at any concentration. The addition of ionic compounds NaL, SLEC, LTAB, and NaSal caused a significant decrease with increasing concentration. Building on the research of Jonas Blahnik⁹⁰ and Marcus *et al.*⁵⁵, the impact of an additive on the binodal is proposed to depend on the presence or absence of charge, their solubility in the SFME solvent compounds, and, in the case of an ionic surfactant, the hydration of the headgroup. This conclusion was derived from three borderline cases: nonionic surfactants C12E10 and GML are surface-active without bearing charges and are soluble in EtOH, water, and slightly in Oct. This combination resulted in slightly larger pre-Ouzo aggregates and an extension of the BIC phase toward higher % Oct at 7.5% C12E10. It was assumed that the high solubility in EtOH and water reduced interfacial affinity, resulting in only minor stabilization of the present mesostructures. NaSal is a surface-active salt that is soluble in EtOH and water, but less soluble in Oct than C12E10. Despite reduced interfacial affinity due to its solubility in EtOH and water, little charging of the interfaces stabilizes the present mesostructures to a greater extent than with nonionic surfactants. This results in the formation of significantly larger pre-Ouzo aggregates, a more pronounced extension of the BIC phase, and a reduction of the binodal due to ion hydration. Maximum decrease in the binodal was suggested to result from a combination of surface activity and the presence of a charge, reduced solubility in the hydrotrope, and strong hydration. This was the case for the tested anionic surfactants. Their addition led to the formation of smaller pre-Ouzo aggregates at low % Oct, which increased in size with increasing % Oct. Additionally, the pre-Ouzo region was found to be extended to higher % Oct, thus being favored over the former BIC phase. Increased charging due to high interfacial affinity and strong hydration is suggested to be responsible for significantly decreasing the binodal, even at small additive concentrations, and to shift the critical point to higher % Oct. For generalization, testing with other compounds and other SFMEs is necessary. The proposed concept could be interesting for future applications in which reducing organic solvents and increasing water content in formulations are desirable. By stabilizing the mesostructures in SFMEs, a connection could also be made to applications that use the stable, monodisperse compartmentation of classical μ Es.

4.6. References

- (1) Dörfler, H.-D. *Grenzflächen und kolloid-disperse Systeme*; Springer: Berlin, **2002**. ISBN 3-540-42547-0.
- (2) Holmberg, K. Surfactants. *Ullmann's Encyclopedia of Industrial Chemistry*; Wiley-VCH Verlag: Weinheim, **2019**; pp 1–56. https://doi.org/10.1002/14356007.a25_747.pub2.
- (3) Evans, D. F.; Wennerström, H., Eds. *The Colloidal Domain: Where Physics, Chemistry, Biology, and Technology Meet*, second edition; Wiley-VCH: Weinheim, **1999**. ISBN 978-0-471-24247-5.
- (4) Holmberg, K., Ed. *Handbook of Applied Surface and Colloidal Chemistry*, Volume 2; John Wiley & Sons: Chichester, **2002**. ISBN 0-471-49083-0.
- (5) Langevin, D. Micelles and Microemulsions. *Annu. Rev. Phys. Chem.* **1992**, *43*, 341–369. <https://doi.org/10.1146/annurev.pc.43.100192.002013>.
- (6) Mihailescu, E.; Monkenbusch, M.; Endo, H.; Allgaier, J.; Gompper, G.; Stellbrink, J.; Richter, D.; Jakobs, B.; Sottmann, T.; Farago, B. Dynamics of bicontinuous microemulsion phases with and without amphiphilic block-copolymers. *J. Chem. Phys.* **2001**, *115*, 9563–9577. <https://doi.org/10.1063/1.1413509>.
- (7) Krickl, S. Investigation of Potential Applications of Surfactant-Free Microemulsions - Solubilisation, Separation and Extraction Processes and Reaction Media. Dissertation, University of Regensburg, Regensburg, **2018**. <https://doi.org/10.5283/epub.38134>.
- (8) Tartaro, G.; Mateos, H.; Schirone, D.; Angelico, R.; Palazzo, G. Microemulsion Microstructure(s): A Tutorial Review. *Nanomaterials* **2020**, *10*, 1657–1697. <https://doi.org/10.3390/nano10091657>.
- (9) Strey, R. Microemulsion microstructure and interfacial curvature. *Colloid Polym. Sci.* **1994**, *272*, 1005–1019. <https://doi.org/10.1007/BF00658900>.
- (10) de Gennes, P. G.; Taupin, C. Microemulsions and the flexibility of oil/water interfaces. *J. Phys. Chem.* **1982**, *86*, 2294–2304. <https://doi.org/10.1021/j100210a011>.
- (11) Prévost, S.; Gradzielski, M.; Zemb, T. Self-assembly, phase behaviour and structural behaviour as observed by scattering for classical and non-classical microemulsions. *Adv. Colloid Interface Sci.* **2017**, *247*, 374–396. <https://doi.org/10.1016/j.cis.2017.07.022>.
- (12) Myers, D. *Surfactant Science and Technology*, Third edition; Wiley-Interscience: New York, **2006**. <https://doi.org/10.1002/047174607X.ch5>.
- (13) Orlich, B.; Schomäcker, R. Enzyme Catalysis in Reverse Micelles. In *History and Trends in Bioprocessing and Biotransformation*; Dutta, N. N., Hammar, F., Haralampidis, K., Karanth, N. G., König, A., Krishna, S. H., Kunze, G., Nagy, E., Orlich, B., Osbourn, A. E., Raghavarao, K. S. M. S., Riedel, K., Sahoo, G. C., Schomäcker, R., Srinivas, N. D., Trojanowska, M., Eds.; Springer: Berlin, Heidelberg, **2002**; pp 185–208. https://doi.org/10.1007/3-540-44604-4_6.

- (14) Brüss, L.; Jeyaseelan, R.; Kürschner, J.; Utikal, M.; Næsborg, L. Micellar Effects and their Relevance in Photochemistry and Photocatalysis. *ChemCatChem* **2022**, *15*, 1–8. <https://doi.org/10.1002/cctc.202201146>.
- (15) Giedyk, M.; Narobe, R.; Weiß, S.; Touraud, D.; Kunz, W.; König, B. Photocatalytic activation of alkyl chlorides by assembly-promoted single electron transfer in microheterogeneous solutions. *Nat. Catal.* **2020**, *3*, 1–8. <https://doi.org/10.1038/s41929-019-0369-5>.
- (16) Shen, T.; Zhou, S.; Ruan, J.; Chen, X.; Liu, X.; Ge, X.; Qian, C. Recent advances on micellar catalysis in water. *Adv. Colloid Interface Sci.* **2021**, *287*, 102299–102314. <https://doi.org/10.1016/j.cis.2020.102299>.
- (17) Candau, F.; Selb, J. Hydrophobically-modified polyacrylamides prepared by micellar polymerization1Part of this paper was presented at the conference on ‘Associating Polymer’, Fontevraud, France, November 1997.1. *Adv. Colloid Interface Sci.* **1999**, *79*, 149–172. [https://doi.org/10.1016/S0001-8686\(98\)00077-3](https://doi.org/10.1016/S0001-8686(98)00077-3).
- (18) Liu, Y.; Goebel, J.; Yin, Y. Templated synthesis of nanostructured materials. *Chem. Soc. Rev.* **2013**, *42*, 2610–2653. <https://doi.org/10.1039/C2CS35369E>.
- (19) Lawrence, M. J.; Rees, G. D. Microemulsion-based media as novel drug delivery systems. *Adv. Drug Deliv. Rev.* **2000**, *45*, 89–121. [https://doi.org/10.1016/S0169-409X\(00\)00103-4](https://doi.org/10.1016/S0169-409X(00)00103-4).
- (20) Scott, M. J.; Jones, M. N. The biodegradation of surfactants in the environment. *Biochim. Biophys. Acta* **2000**, *1508*, 235–251. [https://doi.org/10.1016/S0304-4157\(00\)00013-7](https://doi.org/10.1016/S0304-4157(00)00013-7).
- (21) Jarda, K.; Droguet, P.; Daghrir, R. Surfactants in aquatic and terrestrial environment: occurrence, behavior, and treatment processes. *Environ. Sci. Pollut. Res. Int.* **2016**, *23*, 3195–3216. <https://doi.org/10.1007/s11356-015-5803-x>.
- (22) Mehninger, J.; Kunz, W. Carl Neuberg’s hydrotropic appearances (1916). *Adv. Colloid Interface Sci.* **2021**, *294*, 102476–102482. <https://doi.org/10.1016/j.cis.2021.102476>.
- (23) Neuberg, C. Hydrotropische Erscheinungen. *Biochem. Z.* **1916**, *76*, 107–108.
- (24) Kunz, W.; Holmberg, K.; Zemb, T. Hydrotropes. *Curr. Opin. Colloid Interface Sci.* **2016**, *22*, 99–107. <https://doi.org/10.1016/j.cocis.2016.03.005>.
- (25) Bauduin, P.; Renoncourt, A.; Kopf, A.; Touraud, D.; Kunz, W. Unified Concept of Solubilization in Water by Hydrotropes and Cosolvents. *Langmuir* **2005**, *21*, 6769–6775. <https://doi.org/10.1021/la050554l>.
- (26) Klossek, M. L.; Touraud, D.; Zemb, T.; Kunz, W. Structure and Solubility in Surfactant-Free Microemulsions. *ChemPhysChem* **2012**, *13*, 4116–4119. <https://doi.org/10.1002/cphc.201200667>.
- (27) Zemb, T. N.; Klossek, M.; Lopian, T.; Marcus, J.; Schöettl, S.; Horinek, D.; Prevost, S. F.; Touraud, D.; Diat, O.; Marčelja, S.; *et al.* How to explain microemulsions formed by solvent mixtures without conventional surfactants. *Proc. Natl. Acad. Sci.* **2016**, *113*, 4260–4265. <https://doi.org/10.1073/pnas.1515708113>.
- (28) Lopian, T.; Schöettl, S.; Prevost, S.; Pellet-Rostaing, S.; Horinek, D.; Kunz, W.; Zemb, T. Morphologies Observed in Ultraflexible Microemulsions with and without the Presence of a Strong Acid. *ACS Cent. Sci.* **2016**, *2*, 467–475. <https://doi.org/10.1021/acscentsci.6b00116>.

- (29) Schöttl, S.; Horinek, D. Aggregation in detergent-free ternary mixtures with microemulsion-like properties. *Curr. Opin. Colloid Interface Sci.* **2016**, *22*, 8–13. <https://doi.org/10.1016/j.cocis.2016.02.003>.
- (30) Vitale, S. A.; Katz, J. L. Liquid Droplet Dispersions Formed by Homogeneous Liquid–Liquid Nucleation: “The Ouzo Effect”. *Langmuir* **2003**, *19*, 4105–4110. <https://doi.org/10.1021/la026842o>.
- (31) Lu, Z.; Xu, H.; Zeng, H.; Zhang, X. Solvent Effects on the Formation of Surface Nanodroplets by Solvent Exchange. *Langmuir* **2015**, *31*, 12120–12125. <https://doi.org/10.1021/acs.langmuir.5b03303>.
- (32) Ganachaud, F.; Katz, J. L. Nanoparticles and nanocapsules created using the Ouzo effect: spontaneous emulsification as an alternative to ultrasonic and high-shear devices. *ChemPhysChem* **2005**, *6*, 209–216. <https://doi.org/10.1002/cphc.200400527>.
- (33) Prévost, S.; Krickl, S.; Marčelja, S.; Kunz, W.; Zemb, T.; Grillo, I. Spontaneous Ouzo Emulsions Coexist with Pre-Ouzo Ultraflexible Microemulsions. *Langmuir* **2021**, *37*, 3817–3827. <https://doi.org/10.1021/acs.langmuir.0c02935>.
- (34) Blahnik, J.; Müller, E.; Braun, L.; Denk, P.; Kunz, W. Nanoscopic microheterogeneities or pseudo-phase separations in non-conventional liquids. *Curr. Opin. Colloid Interface Sci.* **2022**, *57*, 101535–101548. <https://doi.org/10.1016/j.cocis.2021.101535>.
- (35) Schubert, K.-V.; Strey, R.; Kline, S. R.; Kaler, E. W. Small angle neutron scattering near Lifshitz lines: Transition from weakly structured mixtures to microemulsions. *J. Chem. Phys.* **1994**, *101*, 5343–5355. <https://doi.org/10.1063/1.467387>.
- (36) Diat, O.; Klossek, M. L.; Touraud, D.; Deme, B.; Grillo, I.; Kunz, W.; Zemb, T. Octanol-rich and water-rich domains in dynamic equilibrium in the pre-ouzo region of ternary systems containing a hydrotrope. *J. Appl. Cryst.* **2013**, *46*, 1665–1669. <https://doi.org/10.1107/S002188981302606X>.
- (37) Prévost, S.; Lopian, T.; Pleines, M.; Diat, O.; Zemb, T. Small-angle scattering and morphologies of ultra-flexible microemulsions. *J. Appl. Cryst.* **2016**, *49*, 2063–2072. <https://doi.org/10.1107/s1600576716016150>.
- (38) Schöttl, S.; Lopian, T.; Prévost, S.; Touraud, D.; Grillo, I.; Diat, O.; Zemb, T.; Horinek, D. Combined molecular dynamics (MD) and small angle scattering (SAS) analysis of organization on a nanometer-scale in ternary solvent solutions containing a hydrotrope. *J. Colloid Interface Sci.* **2019**, *540*, 623–633. <https://doi.org/10.1016/j.jcis.2019.01.037>.
- (39) Schöttl, S.; Marcus, J.; Diat, O.; Touraud, D.; Kunz, W.; Zemb, T.; Horinek, D. Emergence of surfactant-free micelles from ternary solutions. *Chem. Sci.* **2014**, *5*, 2949–2954. <https://doi.org/10.1039/C4SC00153B>.
- (40) Subramanian, D.; Anisimov, M. A. Phase behavior and mesoscale solubilization in aqueous solutions of hydrotropes. *Fluid Phase Equilib.* **2014**, *362*, 170–176. <https://doi.org/10.1016/j.fluid.2013.09.064>.
- (41) Rak, D.; Sedlák, M. On the Mesoscale Solubility in Liquid Solutions and Mixtures. *J. Phys. Chem. B* **2019**, *123*, 1365–1374. <https://doi.org/10.1021/acs.jpcc.8b10638>.

- (42) Borys, N. F.; Holt Smith, L.; Barden, R. E. Detergentless water/oil microemulsions. III. Effect of KOH on phase diagram and effect of solvent composition on base hydrolysis of esters. *J. Colloid Interface Sci.* **1979**, *71*, 526–532. [https://doi.org/10.1016/0021-9797\(79\)90327-8](https://doi.org/10.1016/0021-9797(79)90327-8).
- (43) Keiser, B. A.; Varie, D.; Barden, R. E.; Holt Smith, L. Detergentless water/oil microemulsions composed of hexane, water, and 2-propanol. 2. Nuclear magnetic resonance studies, effect of added sodium chloride. *J. Phys. Chem.* **1979**, *83*, 1276–1280. <https://doi.org/10.1021/j100473a008>.
- (44) Lund, G.; Holt, S. L. Detergentless water/oil microemulsions: IV. the ternary pseudo-phase diagram for and properties of the system toluene/2-propanol/water. *J. Am. Oil Chem. Soc.* **1980**, *57*, 264–267. <https://doi.org/10.1007/BF02668258>.
- (45) Smith, G. D.; Donelan, C. E.; Barden, R. E. Oil-continuous microemulsions composed of hexane, water, and 2-propanol. *J. Colloid Interface Sci.* **1977**, *60*, 488–496. [https://doi.org/10.1016/0021-9797\(77\)90313-7](https://doi.org/10.1016/0021-9797(77)90313-7).
- (46) Abbott, S.; Booth, J. J.; Shimizu, S. Practical molecular thermodynamics for greener solution chemistry. *Green Chem.* **2017**, *19*, 68–75. <https://doi.org/10.1039/C6GC03002E>.
- (47) Schöttl, S.; Matubayasi, N.; Horinek, D. Solubilization power of surfactant-free microemulsions. *Phys. Chem. Chem. Phys.* **2020**, *22*, 22185–22189. <https://doi.org/10.1039/D0CP02933E>.
- (48) Fan, T.; Chen, L.; Xia, X.; Wu, Y.; Zhang, J.; Yin, K.; Liu, F.; Yan, Z. Dissipative Particle Dynamics Quantitative Simulation of the Formation Mechanism and Emulsification Driving Force of Deep Eutectic Solvent-Based Surfactant-Free and Water-Free Microemulsion. *Ind. Eng. Chem. Res.* **2021**, *60*, 3249–3258. <https://doi.org/10.1021/acs.iecr.0c06193>.
- (49) Han, Y.; Pan, N.; Li, D.; Liu, S.; Sun, B.; Chai, J. Formation mechanism of surfactant-free microemulsion and a judgment on whether it can be formed in one ternary system. *Chem. Eng. J.* **2022**, *437*, 135385–135396. <https://doi.org/10.1016/j.cej.2022.135385>.
- (50) Stemplinger, S.; Zemb, T.; Horinek, D.; Dufrêche, J.-F. Aggregating fluids under centrifugal fields. *J. Mol. Liq.* **2023**, *386*, 122358–122368. <https://doi.org/10.1016/j.molliq.2023.122358>.
- (51) Seo, S.; Lee, H.-S.; Yoon, T. J. Kirkwood-Buff Analysis of Binary and Ternary Systems Consisting of Alcohols (Methanol, Ethanol, 1-Propanol, and 2-Propanol), Water, and n-Hexane to Understand the Formation of Surfactant-Free Microemulsions. *J. Phys. Chem. B* **2024**, *128*, 5092–5108. <https://doi.org/10.1021/acs.jpcc.4c01563>.
- (52) Chen, C.; Gao, Y.; Li, M.; Sun, C. Unraveling the Phase Behavior and Stability of Surfactant-Free Microemulsions: From Molecular Interactions to Macroscopic Properties. *Langmuir* **2024**, *40*, 26227–26238. <https://doi.org/10.1021/acs.langmuir.4c03761>.
- (53) Ma, W.; Du, N.; Hou, W. Thermodynamic Explanation of Surfactant-Free Microemulsions. *J. Phys. Chem. B* **2025**, *129*, 2115–2127. <https://doi.org/10.1021/acs.jpcc.4c08376>.
- (54) Zemb, T.; Kunz, W. Weak aggregation: State of the art, expectations and open questions. *Curr. Opin. Colloid Interface Sci.* **2016**, *22*, 113–119. <https://doi.org/10.1016/j.cocis.2016.04.002>.

- (55) Marcus, J.; Touraud, D.; Prévost, S.; Diat, O.; Zemb, T.; Kunz, W. Influence of additives on the structure of surfactant-free microemulsions. *Phys. Chem. Chem. Phys.* **2015**, *17*, 32528–32538. <https://doi.org/10.1039/C5CP06364G>.
- (56) Onuki, A. Surface tension of electrolytes: Hydrophilic and hydrophobic ions near an interface. *J. Chem. Phys.* **2008**, *128*, 224704-224713. <https://doi.org/10.1063/1.2936992>.
- (57) Onuki, A.; Yabunaka, S.; Araki, T.; Okamoto, R. Structure formation due to antagonistic salts. *Curr. Opin. Colloid Interface Sci.* **2016**, *22*, 59–64. <https://doi.org/10.48550/arXiv.1604.00598>.
- (58) Winkler, R.; Buchecker, T.; Hastreiter, F.; Touraud, D.; Kunz, W. PPh4Cl in aqueous solution – the aggregation behavior of an antagonistic salt. *Phys. Chem. Chem. Phys.* **2017**, *19*, 25463–25470. <https://doi.org/10.1039/C7CP02677C>.
- (59) Schöttl, S.; Horinek, D. Salt effects in surfactant-free microemulsions. *J. Chem. Phys.* **2018**, *148*, 222818-222824. <https://doi.org/10.1063/1.5022883>.
- (60) Gao, Y.; Chen, C.; Li, M.; Sun, C. Ionic environment-modulated nucleation and stability of multiscale nanodomains in surfactant-free microemulsions. *J. Colloid Interface Sci.* **2025**, *696*, 137833-137844. <https://doi.org/10.1016/j.jcis.2025.137833>.
- (61) Xenakis, A.; Zoumpanti, M.; Stamatis, H. Enzymatic reactions in structured surfactant-free microemulsions. *Curr. Opin. Colloid Interface Sci.* **2016**, *22*, 41–45. <https://doi.org/10.1016/j.cocis.2016.02.009>.
- (62) Zoumpanti, M.; Karali, M.; Xenakis, A.; Stamatis, H. Lipase biocatalytic processes in surfactant free microemulsion-like ternary systems and related organogels. *Enzym. Microb. Technol.* **2006**, *39*, 531–539. <https://doi.org/10.1016/j.enzmictec.2005.03.030>.
- (63) O'Connor, C. J.; Petricevic, S. F.; Coddington, J. M.; Stanley, R. A. An NMR assay for quantitating lipase activity in biphasic macroemulsions. *J. Am. Oil Chem. Soc.* **1992**, *69*, 295–300. <https://doi.org/10.1007/BF02636053>.
- (64) Khmelnitsky, Y. L.; Hilhorst, R.; Veeger, C. Detergentless microemulsions as media for enzymatic reactions. Cholesterol oxidation catalyzed by cholesterol oxidase. *Eur. J. Biochem.* **1988**, *176*, 265–271. <https://doi.org/10.1111/j.1432-1033.1988.tb14277.x>.
- (65) Hofmann, E. The Interplay of Surfactant-Free Mesoscopic Structuring and (Bio-)Catalysis in Water, Universität Regensburg, **2024**. <http://doi.org/10.5283/epub.54624>.
- (66) Hofmann, E.; Schmauser, L.; Neugebauer, J.; Touraud, D.; Gallou, F.; Kunz, W. Sustainable cascade reaction combining transition metal-biocatalysis and hydrophobic substrates in surfactant-free aqueous solutions. *Chem. Eng. J.* **2023**, *472*, 144599-144608. <https://doi.org/10.1016/j.cej.2023.144599>.
- (67) Schmauser, L.; Müller, E.; Touraud, D.; Kunz, W. Applications of surfactant-free microemulsions. *Curr. Opin. Colloid Interface Sci.*, **2026**, *83*, 102001-102018. <https://doi.org/10.1016/j.cocis.2026.102001>.
- (68) Degot, P.; Huber, V.; Hofmann, E.; Hahn, M.; Touraud, D.; Kunz, W. Solubilization and extraction of curcumin from Curcuma Longa using green, sustainable, and food-approved surfactant-free microemulsions. *Food Chem.* **2021**, *336*, 127660-127668. <https://doi.org/10.1016/j.foodchem.2020.127660>.

- (69) Degot, P.; Huber, V.; Touraud, D.; Kunz, W. Curcumin extracts from *Curcuma Longa* – Improvement of concentration, purity, and stability in food-approved and water-soluble surfactant-free microemulsions. *Food Chem.* **2021**, *339*, 128140–128148. <https://doi.org/10.1016/j.foodchem.2020.128140>.
- (70) Zhang, X.; Song, M.; Chai, J.; Cui, X.; Wang, J. Preparation, characterization and application of a surfactant-free microemulsion containing 1-octen-3-ol, ethanol, and water. *J. Mol. Liq.* **2020**, *300*, 112278–112288. <https://doi.org/10.1016/j.molliq.2019.112278>.
- (71) Marcus, J.; Müller, M.; Nistler, J.; Touraud, D.; Kunz, W. Nano-droplet formation in water/ethanol or isopropanol/mosquito repellent formulations. *Colloids Surf. A Physicochem. Eng. Asp.* **2014**, *458*, 3–9. <https://doi.org/10.1016/j.colsurfa.2013.11.030>.
- (72) Marcus, J.; Klossek, M.; Touraud, D.; Kunz, W. Nano-droplet formation in fragrance tinctures. *Flavour Fragr. J.* **2013**, *28*, 294–299. <https://doi.org/10.1002/ffj.3172>.
- (73) Cui, X.; Wang, J.; Zhang, X.; Wang, Q.; Song, M.; Chai, J. Preparation of Nano-TiO₂ by a Surfactant-Free Microemulsion–Hydrothermal Method and Its Photocatalytic Activity. *Langmuir* **2019**, *35*, 9255–9263. <https://doi.org/10.1021/acs.langmuir.9b01392>.
- (74) Han, Y.; Pan, N.; Liu, S.; Chai, J.; Li, D. Growth of nano metal oxide in surfactant-free microemulsion template and its catalytic mechanism simulation. *J. Environ. Chem. Eng.* **2022**, *10*, 108006–108007. <https://doi.org/10.1016/j.jece.2022.108006>.
- (75) Lu, J.; Peng, L.; Zhang, A.; Xu, J.; Wu, M.; Ma, S. Fabrication of tetraethyl orthosilicate/ethanol–water surfactant-free microemulsions and their applications in self-templating synthesis of monodispersed silica colloidal spheres. *RSC Adv.* **2024**, *14*, 39040–39049. <https://doi.org/10.1039/D4RA07679F>.
- (76) Sun, B.; Chai, J.; Chai, Z.; Zhang, X.; Cui, X.; Lu, J. A surfactant-free microemulsion consisting of water, ethanol, and dichloromethane and its template effect for silica synthesis. *J. Colloid Interface Sci.* **2018**, *526*, 9–17. <https://doi.org/10.1016/j.jcis.2018.04.072>.
- (77) Blahnik, J.; Krickl, S.; Schmid, K.; Müller, E.; Lupton, J.; Kunz, W. Microemulsion and microsuspension polymerization of methyl methacrylate in surfactant-free microemulsions (SFME). *J. Colloid Interface Sci.* **2023**, *648*, 755–767. <https://doi.org/10.1016/j.jcis.2023.06.025>.
- (78) Blahnik, J.; Schuster, J.; Müller, R.; Müller, E.; Kunz, W. Surfactant-free microemulsions (SFMEs) as a template for porous polymer synthesis. *J. Colloid Interface Sci.* **2024**, *655*, 371–382. <https://doi.org/10.1016/j.jcis.2023.10.162>.
- (79) Clause, M.; Nicholas-Morgantini, L.; Zradba, A.; Touraud, D. Water/Ionic Surfactant/Alkanol/Hydrocarbon Systems: Influence of Certain Constitution and Composition Parameters upon the Realms-of-Existence and Transport Properties of Microemulsion-Type Media. In *Surfactant Science Series*; Dekker M. (Hg.), New York, **1987**; pp 15–62.
- (80) Hassan, P. A.; Rana, S.; Verma, G. Making sense of Brownian motion: colloid characterization by dynamic light scattering. *Langmuir* **2015**, *31*, 3–12. <https://doi.org/10.1021/la501789z>.
- (81) Stetefeld, J.; McKenna, S. A.; Patel, T. R. Dynamic light scattering: a practical guide and applications in biomedical sciences. *Biophys. Rev.* **2016**, *8*, 409–427. <https://doi.org/10.1007/s12551-016-0218-6>.

- (82) Øgden, L. *Light Scattering Demystified: Theory and Practice*, **2017**.
- (83) Frisken, B. J. Revisiting the method of cumulants for the analysis of dynamic light-scattering data. *Appl. Opt.* **2001**, *40*, 4087–4091. <https://doi.org/10.1364/ao.40.004087>.
- (84) Narayanan, T.; Sztucki, M.; Zinn, T.; Kieffer, J.; Homs-Puron, A.; Gorini, J.; van Vaerenbergh, P.; Boesecke, P. Performance of the time-resolved ultra-small-angle X-ray scattering beamline with the Extremely Brilliant Source. *J. Appl. Cryst.* **2022**, *55*, 98–111. <https://doi.org/10.1107/s1600576721012693>.
- (85) Prévost, S.; Zemb, T. Chapter 25 - Using Weak Aggregation for Solubilization and Separation. In *Neutrons, X-rays, and Light (Second Edition)*; Lindner, P., Oberdisse, J., Eds.; Elsevier, **2025**; pp 701–740. <https://doi.org/10.1016/B978-0-443-29116-6.00005-9>.
- (86) Ornstein, L. S.; Zernike, F. Accidental Deviations of Density and Opalescence at the Critical Point of a Single Substance. *Proc. Akad. Sci.* **1914**, *XVII*, 793–806.
- (87) Pensini, E.; Marangoni, A. G.; Bartokova, B.; Fameau, A. L.; Corradini, M. G.; Stobbs, J. A.; Arthur, Z.; Prévost, S. Sulfolane clustering in aqueous saline solutions. *Phys. Fluids* **2024**, *36*, 37117–37130. <https://doi.org/10.1063/5.0196389>.
- (88) Teubner, M.; Strey, R. Origin of the scattering peak in microemulsions. *J. Chem. Phys.* **1987**, *87*, 3195–3200. <https://doi.org/10.1063/1.453006>.
- (89) Denk, P.; Reigl, S.; Rödig, B.; Sztucki, M.; Prévost, S.; Zemb, T.; Kunz, W. Biliquid oil-in-water nanofoams and spontaneous emulsification obtained with a surfactant resistant to curvature changes. *J. Colloid Interface Sci.* **2025**, *684*, 105–121. <https://doi.org/10.1016/j.jcis.2025.01.090>.
- (90) Blahnik, J. Polymerizations in Surfactant-free Mesostuctured Liquids. Dissertation, University of Regensburg, Regensburg, **2023**. <https://doi.org/10.5283/epub.55333>.
- (91) Chiappisi, L. Polyoxyethylene alkyl ether carboxylic acids: An overview of a neglected class of surfactants with multiresponsive properties. *Adv. Colloid Interface Sci.* **2017**, *250*, 79–94. <https://doi.org/10.1016/j.cis.2017.10.001>.
- (92) Denk, P.; El Maangar, A.; Lal, J.; Kleber, D.; Zemb, T.; Kunz, W. Phase diagrams and microstructures of aqueous short alkyl chain polyethylene glycol ether carboxylate and carboxylic acid triblock surfactant solutions. *J. Colloid Interface Sci.* **2021**, *590*, 375–386. <https://doi.org/10.1016/j.jcis.2021.01.061>.
- (93) Denk, P.; El Maangar, A.; Prévost, S.; Silva, W.; Gschwind, R.; Zemb, T.; Kunz, W. Cloud point, auto-coacervation, and nematic ordering of micelles formed by ethylene oxide containing carboxylate surfactants. *J. Colloid Interface Sci.* **2022**, *621*, 470–488. <https://doi.org/10.1016/j.jcis.2022.04.046>.
- (94) Vlachy, N.; Jagoda-Cwiklik, B.; Vácha, R.; Touraud, D.; Jungwirth, P.; Kunz, W. Hofmeister series and specific interactions of charged headgroups with aqueous ions. *Adv. Colloid Interface Sci.* **2009**, *146*, 42–47. <https://doi.org/10.1016/j.cis.2008.09.010>.
- (95) Mehrlinger, J.; Hofmann, E.; Touraud, D.; Koltzenburg, S.; Kellermeier, M.; Kunz, W. Salting-in and salting-out effects of short amphiphilic molecules: a balance between specific ion effects and hydrophobicity. *Phys. Chem. Chem. Phys.* **2021**, *23*, 1381–1391. <https://doi.org/10.1039/D0CP05491G>.

- (96) Kunz, W. Specific ion effects in colloidal and biological systems. *Curr. Opin. Colloid Interface Sci.* **2010**, *15*, 34–39. <https://doi.org/10.1016/j.cocis.2009.11.008>.
- (97) Zana, R. Aqueous surfactant-alcohol systems: A review. *Adv. Colloid Interface Sci.* **1995**, *57*, 1–64. [https://doi.org/10.1016/0001-8686\(95\)00235-1](https://doi.org/10.1016/0001-8686(95)00235-1).
- (98) Campbell, A. N.; Lakshminarayanan, G. R. Conductances and surface tensions of aqueous solutions of sodium decanoate, sodium laurate, and sodium myristate at 25 and 35. *Can. J. Chem.* **1965**, *43*, 1729–1737. <https://doi.org/10.1139/v65-228>.
- (99) Chauhan, S.; Kaur, M.; Kumar, K.; Chauhan, M. S. Study of the effect of electrolyte and temperature on the critical micelle concentration of dodecyltrimethylammonium bromide in aqueous medium. *J. Chem. Thermodyn.* **2014**, *78*, 175–181. <https://doi.org/10.1016/j.jct.2014.07.003>.
- (100) Cipiciani, A.; Onori, G.; Savelli, G. Structural properties of water-ethanol mixtures: A correlation with the formation of micellar aggregates. *Chem. Phys. Lett.* **1988**, *143*, 505–509. [https://doi.org/10.1016/0009-2614\(88\)87404-9](https://doi.org/10.1016/0009-2614(88)87404-9).
- (101) Fukuoka, T.; Ikeda, S.; Habe, H.; Sato, S.; Sakai, H.; Abe, M.; Kitamoto, D.; Sakaki, K. Synthesis and Interfacial Properties of Monoacyl Glyceric Acids as a New Class of Green Surfactants. *J. Oleo Sci.* **2012**, *61*, 343–348. <https://doi.org/10.5650/jos.61.343>.
- (102) Hu, C.; Li, R.; Yang, H.; Wang, J. Properties of binary surfactant systems of nonionic surfactants C12E10, C12E23, and C12E42 with a cationic gemini surfactant in aqueous solutions. *J. Colloid Interface Sci.* **2011**, *356*, 605–613. <https://doi.org/10.1016/j.jcis.2011.01.062>.
- (103) Berthod, A.; Tomer, S.; Dorsey, J. G. Polyoxyethylene alkyl ether nonionic surfactants: physicochemical properties and use for cholesterol determination in food. *Talanta* **2001**, *55*, 69–83. [https://doi.org/10.1016/s0039-9140\(01\)00395-2](https://doi.org/10.1016/s0039-9140(01)00395-2).
- (104) Sakai, T.; Ikoshi, R.; Toshida, N.; Kagaya, M. Thermodynamically Stable Vesicle Formation and Vesicle-to-Micelle Transition of Single-Tailed Anionic Surfactant in Water. *J. Phys. Chem. B* **2013**, *117*, 5081–5089. <https://doi.org/10.1021/jp402314f>.
- (105) Flockhart, B. D. The critical micelle concentration of sodium dodecyl sulfate in ethanol-water mixtures. *J. Colloid Sci.* **1957**, *12*, 557–565. [https://doi.org/10.1016/0095-8522\(57\)90061-2](https://doi.org/10.1016/0095-8522(57)90061-2).
- (106) Rao, I. V.; Ruckenstein, E. Micellization behavior in the presence of alcohols. *J. Colloid Interface Sci.* **1986**, *113*, 375–387. [https://doi.org/10.1016/0021-9797\(86\)90173-6](https://doi.org/10.1016/0021-9797(86)90173-6).
- (107) Shinoda, K. The Effect of Chain Length, Salts and Alcohols on the Critical Micelle Concentration. *Bull. Chem. Soc. Jpn.* **1953**, *26*, 101–105. <https://doi.org/10.1246/bcsj.26.101>.
- (108) Almgren, M.; Swarup, S. Size of sodium dodecyl sulfate micelles in the presence of additives i. alcohols and other polar compounds. *J. Colloid Interface Sci.* **1983**, *91*, 256–266. [https://doi.org/10.1016/0021-9797\(83\)90330-2](https://doi.org/10.1016/0021-9797(83)90330-2).
- (109) Suzuki, H. Studies of the effect of ethanol and sodium chloride on the micellization of sodium dodecyl sulfate by gel filtration. *Bull. Chem. Soc. Jpn.* **1976**, *49*, 1470–1474. <https://doi.org/10.1246/bcsj.49.1470>.

- (110) Moya, S. E.; Schulz, P. C. The aggregation of the sodium dodecyl sulfate –n - octanol–water system at low concentration. *Colloid Polym. Sci.* **1999**, 277, 735–742. <https://doi.org/10.1007/s003960050447>.
- (111) Iakovleva, E. A.; Safonova, E. A.; Victorov, A. I. Aggregates of Ethoxylated Surfactant with Added n-Octanol: Details of Corona Structure from a Molecular-Thermodynamic Model. *Fluid Ph. Equilib.* **2021**, 546, 113134–113143. <https://doi.org/10.1016/j.fluid.2021.113134>.
- (112) Malliaris, A. Solubilization of organic molecules in SDS micelles studied by static fluorescence methods. *Adv. Colloid Interface Sci.* **1987**, 27, 153–168. [https://doi.org/10.1016/0001-8686\(87\)85001-7](https://doi.org/10.1016/0001-8686(87)85001-7).
- (113) Buchecker, T.; Krickl, S.; Winkler, R.; Grillo, I.; Bauduin, P.; Touraud, D.; Pfitzner, A.; Kunz, W. The impact of the structuring of hydrotropes in water on the mesoscale solubilisation of a third hydrophobic component. *Phys. Chem. Chem. Phys.* **2017**, 19, 1806–1816. <https://doi.org/10.1039/C6CP06696H>.
- (114) Krickl, S.; Touraud, D.; Bauduin, P.; Zinn, T.; Kunz, W. Enzyme activity of horseradish peroxidase in surfactant-free microemulsions. *J. Colloid Interface Sci.* **2018**, 516, 466–475. <https://doi.org/10.1016/j.jcis.2018.01.077>.
- (115) Ragheb, R.; Nobbmann, U. Multiple scattering effects on intercept, size, polydispersity index, and intensity for parallel (VV) and perpendicular (VH) polarization detection in photon correlation spectroscopy. *Sci. Rep.* **2020**, 10, 21768–21777. <https://doi.org/10.1038/s41598-020-78872-4>.
- (116) D'Arrigo, G.; Teixeira, J. Small-angle neutron scattering study of D2O–alcohol solutions. *J. Chem. Soc., Faraday Trans.* **1990**, 86, 1503–1509. <https://doi.org/10.1039/FT9908601503>.
- (117) Franks, F.; Ives, D. J. G. The structural properties of alcohol–water mixtures. *Q. Rev., Chem. Soc.* **1966**, 20, 1–44. <https://doi.org/10.1039/QR9662000001>.
- (118) Danov, K. D.; Kralchevsky, P. A.; Ananthapadmanabhan, K. P. Micelle–monomer equilibria in solutions of ionic surfactants and in ionic–nonionic mixtures: A generalized phase separation model. *Adv. Colloid Interface Sci.* **2014**, 206, 17–45. <https://doi.org/10.1016/j.cis.2013.02.001>.
- (119) Us'yarov, O. G. Small-angle X-ray scattering in sodium dodecyl sulfate solutions and micelle clustering. *Colloid J.* **2016**, 78, 698–704. <https://doi.org/10.1134/S1061933X16050227>.
- (120) El Maangar, A.; Degot, P.; Huber, V.; Causse, J.; Berthault, P.; Touraud, D.; Kunz, W.; Zemb, T. Pre-nucleation cluster formation upon ethyl acetate addition to an aqueous solution of an anionic hydrotrope. *J. Mol. Liq.* **2020**, 310, 113240–113249. <https://dx.doi.org/10.1016/j.molliq.2020.113240>.
- (121) Degot, P.; Huber, V.; El Maangar, A.; Gramüller, J.; Rohr, L.; Touraud, D.; Zemb, T.; Gschwind, R. M.; Kunz, W. Triple role of sodium salicylate in solubilization, extraction, and stabilization of curcumin from *Curcuma longa*. *J. Mol. Liq.* **2021**, 329, 115538–115549. <https://doi.org/10.1016/j.molliq.2021.115538>.
- (122) Sadakane, K.; Iguchi, N.; Nagao, M.; Endo, H.; Melnichenko, Y. B.; Seto, H. 2D-Ising-like critical behavior in mixtures of water and 3-methylpyridine including antagonistic salt or ionic surfactant. *Soft Matter* **2011**, 7, 1334–1340. <https://doi.org/10.1039/C0SM00598C>.

References

- (123) Sadakane, K.; Seto, H.; Endo, H.; Shibayama, M. A Periodic Structure in a Mixture of D₂O/3-Methylpyridine/NaBPh₄ Induced by Solvation Effect. *J. Phys. Soc. Jpn* **2007**, 76, 113602-113605. <https://doi.org/10.1143/JPSJ.76.113602>.
- (124) Spalla, O. General Theorems in Small-Angle Scattering. In *Neutrons, X-rays, and Light: Scattering Methods Applied to Soft Condensed Matter*, first edition; Lindner, P., Zemb, T., Ed.; Elsevier: Amsterdam, **2002**; pp 49–71. ISBN 978-0-444-51122-5.
- (125) Tchakalova, V.; Hafner, V. Ethanol-free microemulsion perfuming compositions. US9976108B2, **2018**.
- (126) Shick, R. A.; Tucker, C. J.; Piechocki, C.; Gatz, L. A. Ethanol-free Aqueous Perfume Composition. US 2013/0101531 A1, **2013**.
- (127) Behan, J. M.; Ness, J. N.; Traas, P. C.; Vitsas, J. S.; Willis, B. J. Aqueous Perfume Oil Microemulsions. US 5374614A, **1994**.

General Conclusion and Outlook

The work presented combines mechanistic analysis with an emphasis on sustainable applicability across three distinct sub-areas:

In **Chapter 2**, the refunctionalization of commercially available plant protein isolates in aqueous media was examined. Three different isolates from pea (PPIp), chickpea (CPIp), and potato (PoPIp) were investigated. Their relatively low protein solubility, along with DSC, TGA, and SDS-PAGE data, indicated the proteins' denatured state. Various physical and chemical modification methods and their combinations were tested to enhance the aqueous protein solubility and, consequently, their functionality. The pulse protein isolates PPIp and CPIp showed similar solubility behavior, with aggregate hydrophobicity and covalent binding in these as the main factors limiting solubility. PoPIp exhibited different behavior, with increased hydrophobicity and cross-linking proposed compared to PPIp and CPIp. The protein solubility of all tested isolates was significantly improved by targeting both non-covalent and covalent bonds: combining SDS with pH shifting destabilized insoluble aggregates through protein unfolding and increased interaction with SDS. DLS, zeta potential measurements, and SDS-PAGE revealed the formation of strongly negatively charged aggregates. Summarizing the findings, a schematic solubilization mechanism was proposed, which may also apply to other plant protein isolate classes. This study aimed to identify solubility-limiting factors in denatured plant protein isolates by examining individual and combined interactions. No parameter optimization for specific applications was performed, and less emphasis was placed on exact solubility values. It should also be noted that increased solubility does not necessarily lead to better performance in every application. However, it is a crucial first step, and this study can serve as an introduction. By contributing to a deeper understanding of the behavior of commercial plant protein isolates, their potential as environmentally friendly functional agents in nutrition and technology is advanced. In **Chapter 3**, PPIp was used to solubilize the polyphenol curcumin (Cur). By applying a well-established pH shifting method, Cur-loaded PPIp nanoparticles (PPICur) were prepared. Contrary to the consensus in the literature, the binding of Cur to PPI is largely kinetic. While the Cur concentration in solution immediately after preparation was 1.39 ± 0.093 mg/mL, it dropped to only 0.0528 ± 0.0088 mg/mL after 24 hours of constant stirring. Fundamental investigations into supersaturation revealed an underestimated impact of the preparation method of PPICur on Cur binding. SDS-PAGE and SEC data demonstrated the formation of large, soluble protein aggregates, whose size was crucial for Cur binding. The supersaturation and subsequent precipitation of Cur were studied using UV-Vis and fluorescence spectroscopy, which indicated either aggregation of tightly packed Cur molecules on the carrier surface or isomerization and subsequent aggregation of Cur molecules. Changes or degradation

of the protein carrier were largely ruled out, as shown by CD, particle size, and solubility measurements. Based on these findings, the classification of PPICur as a supersaturated drug delivery system (SDDS) was proposed. Summarizing the findings, a schematic solubilization mechanism was proposed, which may, again, also apply to other plant protein isolate classes. This in-depth study has made substantial progress in understanding plant protein carrier systems prepared using a pH-driven method and commercially available products, as well as their ability to bind poorly soluble lipophilic compounds. Emphasizing the formulation's kinetic and stable states enables diverse applications of PPICur in the food and pharmaceutical industries. In **Chapter 4**, the effects of surface-active compounds on the binodal and the mesostructure of the surfactant-free microemulsion (SFME) EtOH/1-Octanol/Water were investigated. Nonionic surfactants C12E10 and GML did not significantly influence the binodal at any concentration. The addition of ionic compounds NaL, SLEC, LTAB, and NaSal caused a significant reduction in the binodal with increasing concentration from 2.5% to 7.5% (w/w) in proportion of EtOH. Building on previous research, the effect of an additive on the binodal was proposed to depend on whether it carries a charge, its solubility in the hydrotrope, and, for ionic surfactants, the hydration of the headgroup. The evaluation of SAXS data revealed changes in the mesostructure upon adding these compounds: while nonionic surfactants slightly stabilized existing mesostructures, the addition of ionic hydrotrope NaSal appears to increase the size of pre-Ouzo aggregates and extend the BIC phase toward higher Oct content. Ionic surfactants caused the most significant reduction in the binodal; on the mesoscale, a shrinkage of pre-Ouzo aggregates at low Oct content was observed, followed by an increase at higher Oct content. Additionally, the pre-Ouzo region was found to extend to a higher Oct content, favoring it over the previous BIC phase. Increased charging due to high interfacial affinity and strong hydration was suggested to not only significantly decrease the binodal, but also to shift the critical point to a higher %Oct. Maintaining the solubilization capacity of a system for a hydrophobic compound while increasing water content and decreasing the organic solvent by adding relatively small amounts of ionic compounds presents an interesting phenomenon with various applications. Understanding how additives impact the mesostructure improves predictability when transitioning into multi-component mixtures in industrial processes.

In summary, two approaches to more sustainable solubilization of hydrophobic components were explored: first, the access and use of environmentally friendly solubilizers, and second, the reduction of organic solvents. Selected examples of both approaches were studied from a fundamental, mechanistic perspective. By developing preliminary schematic models of the ongoing processes and their influencing factors, the understanding and, therefore, the feasibility and predictability for (industrial) applications are improved. In all cases, further research is needed to generalize the proposed concepts, but this work has laid a foundation.

Appendix

Supplementary Information Chapter 2

SDS-PAGE: Composition of the protein loading dye (Laemmli buffer, V=50 mL):

- 15 mL 20% SDS solution
- 12 mL 1.0 M Tris-HCl pH 6.8
- 15 mL glycerol
- 0.03 g bromophenol blue
- DTT is added for a concentration of 0.5 M
- Fill up with water and store at RT
- 150 μ L 2-mercaptoethanol is added to a 1.35 mL aliquot just before use

Table SI 2 1: The aqueous solubilities of the same batch of the used protein isolates at different times and the aqueous solubilities of different batches from the same product at various times.

Protein Isolate	Water solubility [%]		Water solubility [%]	
	(Batch provided by dsm-firmenich)		(Batch gifted from the manufacturer)	
	February 2022	September 2024	February 2023	September 2024
PPIp	16	-	42	32.3 $\pm$ 0.008
CPIp	33	24.7 $\pm$ 0.0009	<i>not available</i>	<i>not available</i>
PoPIp	5	-	-	10.9 $\pm$ 0.002

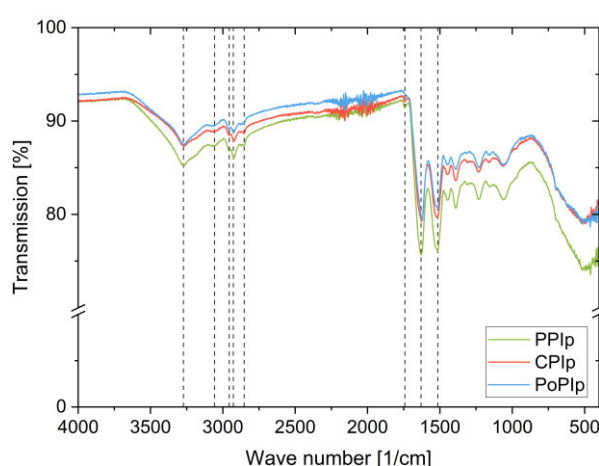


Figure SI 2 1: Infrared spectroscopy of the protein isolates PPIp, CPIp, and PoPIp.

Table SI 2 2: Assignment of the infrared bands of protein isolates PPIp, CPIp, and PoPIp according to Barth 2007¹⁷⁷.

Band position [cm ⁻¹]	Assignment
3271, 3058	v(NH) Amide A and B
2958, 2920, 2852	v(C-H)
1740	v(C=O) Asparagine
1630	v(C=O) Amide I, β -sheet
1513	v(C-N)+v(N-H) Amide II

Table SI 2 3: pH values of the samples prepared from the protein isolates dispersed in aqueous solutions of NaCl, NaSCN, and (NH₄)₂SO₄ in a concentration range between 5 and 100 mM.

Protein Isolate	Sample pH in the additive concentration range 5-100 mM		
	NaCl	NaSCN	(NH ₄) ₂ SO ₄
PPIp	7.0-6.6	6.8-6.6	6.9-6.5
CPIp	6.8-6.5	6.8-6.6	6.6-6.3
PoPIp	5.0-5.1	5.1-5.4	5.1-5.2

Table SI 2 4: pH values of the samples prepared from the protein isolates dispersed in aqueous solutions of SDS, CTAB, and urea in a concentration range between 5 and 100 mM.

Protein Isolate	Sample pH in the additive concentration range 5-100 mM		
	SDS	CTAB	Urea
PPIp	7.4-7.6	6.7-6.1	6.8-6.8
CPIp	7.1-7.4	6.5-5.7	6.9-6.8
PoPIp	6.1-6.3	4.5-4.5	5.0-5.0

Table SI 2 5: pH values of the samples prepared from the protein isolates dispersed in aqueous solutions of VitC in a concentration range between 5 and 100 mM, and 1.7 M.

Protein Isolate	Sample pH in the VitC concentration	
	5-100 mM	1.7 M
PPIp	5.7-3.4	2.0
CPIp	5.8-3.4	2.0
PoPIp	4.2-3.0	1.8

Table SI 2 6: pH values of protein isolates dispersed in aqueous solutions and subjected to pH, temperature, sonication, and addition of Vit C (c=1.7 M) and SDS (c=5-100 mM).

Protein Isolate	Sample pH			
	US+VitC/VitC+T	VitC+T+pH	pH+VitC+T	VitC+SDS
PPIp	2.0	2.0→6.9-7.1	12→2.0	2.0-2.2
CPIp	2.0	2.0→7.0	12→2.0	2.1-2.2
PoPIp	1.8	1.8-2.0→4.9	12→1.8	1.9-2.1

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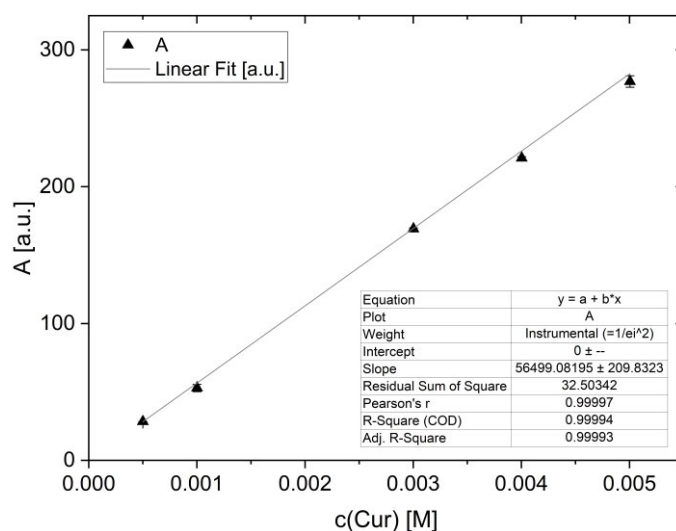


Figure SI 3 1: UV-Vis calibration curve of Cur in EtOH.

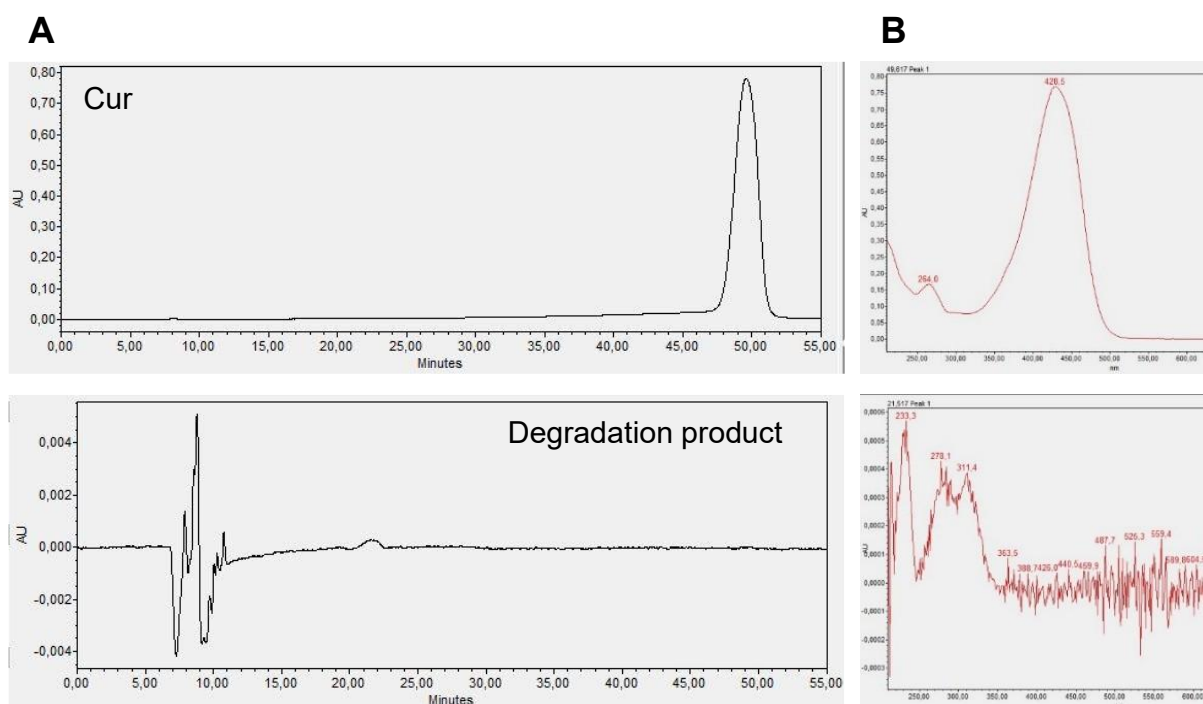


Figure SI 3 2: Chromatograms (A) and UV-Vis absorbance spectra (B) of Cur (49.5 min) and its degradation product vanillin (21.8 min) in EtOH with the respective mobile phases described in the experimental section.

MS: Detailed information on the protocol:

Protein concentration of concentrated SEC-fractions was determined by NanoDrop (Thermo Fisher Scientific) UV-Vis measurement. 10 µg protein was subjected to tryptic digestion using the EasyPep MS Sample Preparation Kit (Thermo Scientific) according to the manufacturer's instructions. Desalted and dried peptides were reconstituted in 0.1% formic acid and applied to LC-MS/MS measurement. Peptide separation was carried out by an UltiMate 3000 RSLCnano System (Thermo Fisher Scientific) equipped with C18 Acclaim Pepman100 preconcentration column (100 µm i.d. x 20 mm, Thermo Fisher Scientific). A linear gradient of 4% to 40% acetonitrile in 0.1% formic acid over 90 min at a flow rate of 300 nl/min was used. The LC-system was on-line coupled to a maXis plus UHR-QTOF system (Bruker Daltonics) via a CaptiveSpray nanoflow electrospray source (Bruker Daltonics). Data-dependent acquisition of MS/MS spectra after collision-induced dissociation (CID) fragmentation was performed at a minimum resolution of 60,000. For the precursor scan, the MS spectra rate was 2 Hz processing a mass range between m/z 175 and m/z 2000. A dynamic method with a fixed cycle time of 3 s was applied via the Compass 1.7 acquisition and processing software (Bruker Daltonics). Raw data processing was performed in Data Analysis 4.2 (Bruker Daltonics) and Protein Scape 3.1.3 (Bruker Daltonics). Mascot 2.5.1 (Matrix Science) was used to perform searching of the Uniprot Pisum sativum database. Search parameters were as follows: enzyme specificity trypsin with 2 missed cleavages allowed, precursor tolerance 0.02 Da, MS/MS tolerance 0.04 Da. Carbamidomethylation modification of cysteine was set as fixed modification, oxidation of methionine, deamidation of asparagine and glutamine, and protein N-terminal acetylation were set as variable modifications. Mascot peptide ion-score cut-off was set to 30. Search conditions were adjusted to provide a false discovery rate of less than 1%.

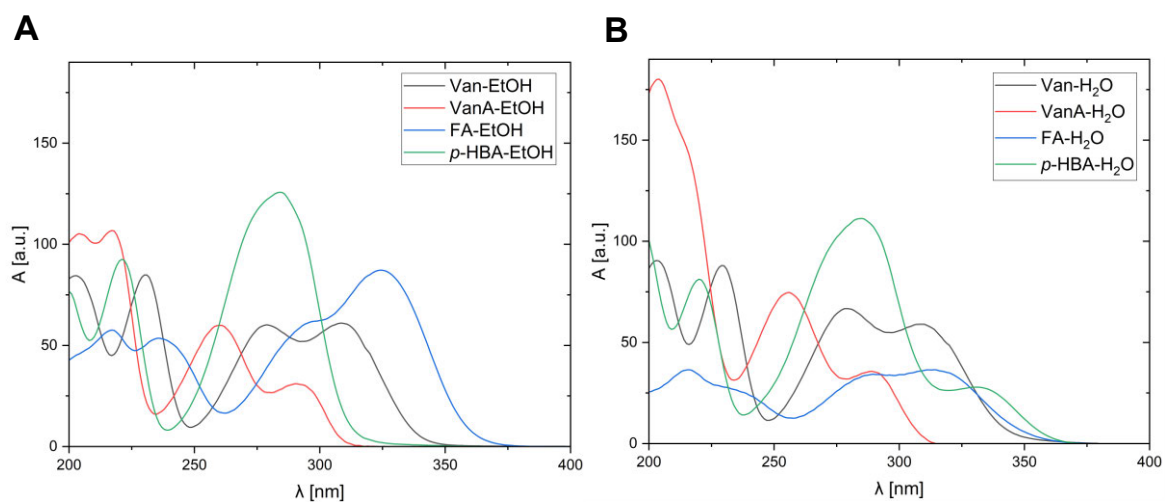


Figure SI 3 3: UV-Vis spectroscopy of Cur degradation products vanillin (Van, $\beta=1$ mg/mL), vanillic acid (VanA, $\beta=1$ mg/mL), ferulic acid (FA, $\beta=1$ mg/mL in EtOH, $\beta=0.5$ mg/mL in water), and *para*-hydroxybenzaldehyde (*p*-HBA, $\beta=1$ mg/mL) in EtOH (A) and water (B).

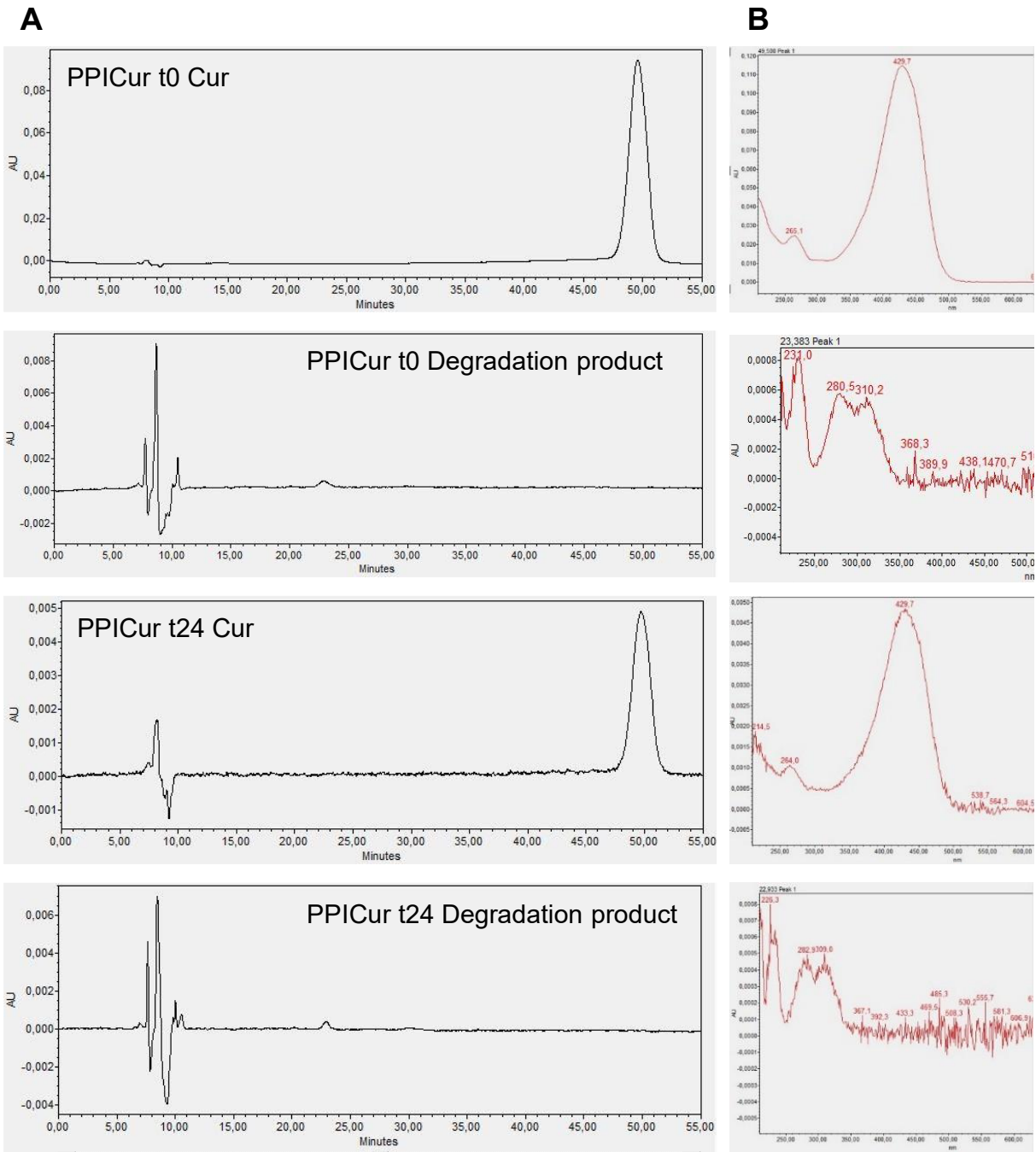


Figure SI 3 4: Chromatograms (A) and UV-Vis absorbance spectra (B) of the ethanolic extracts of PPICur t0 and t24 with peaks at 49.7 min and 22.9 min with the respective mobile phases described in the experimental section.

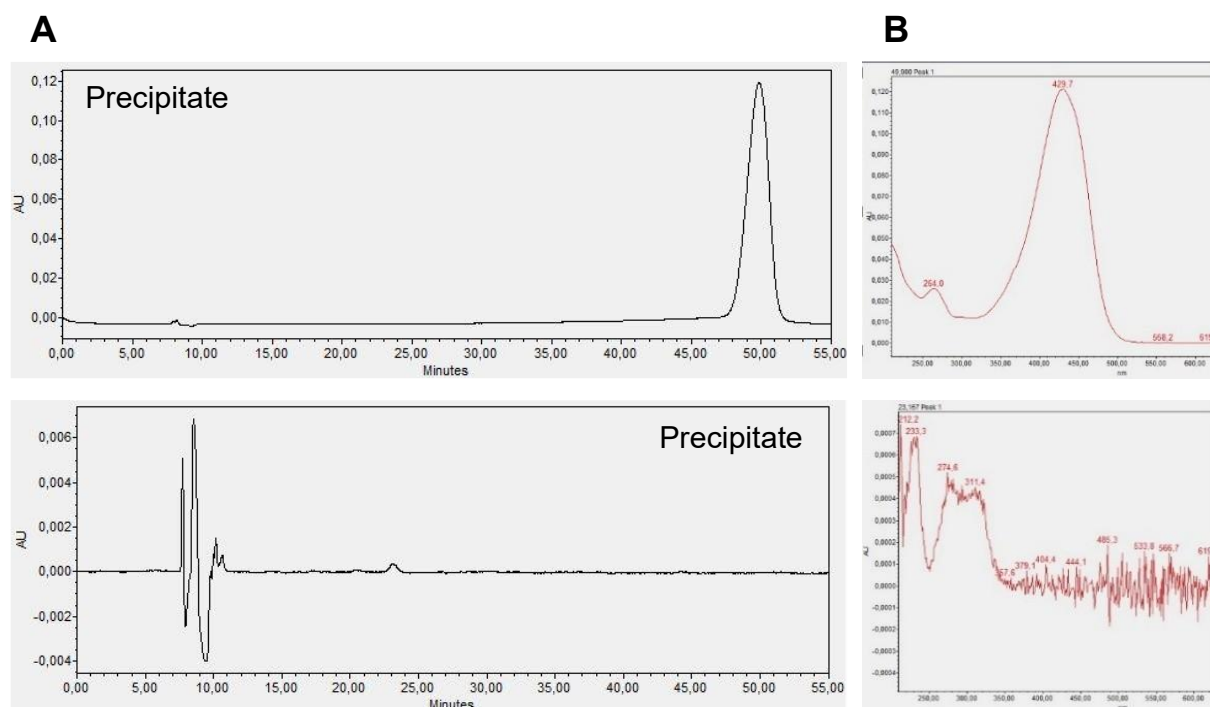


Figure SI 3 5: Chromatograms (**A**) and UV-Vis absorbance spectra (**B**) of the ethanolic extract of the precipitate with peaks at 49.8 min and 23.0 min with the respective mobile phases described in the experimental section.

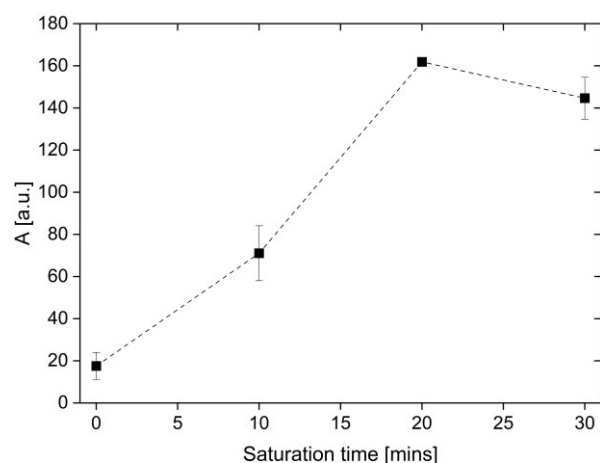


Figure SI 3 6: Determination of the optimal loading time via UV-Vis spectroscopy.

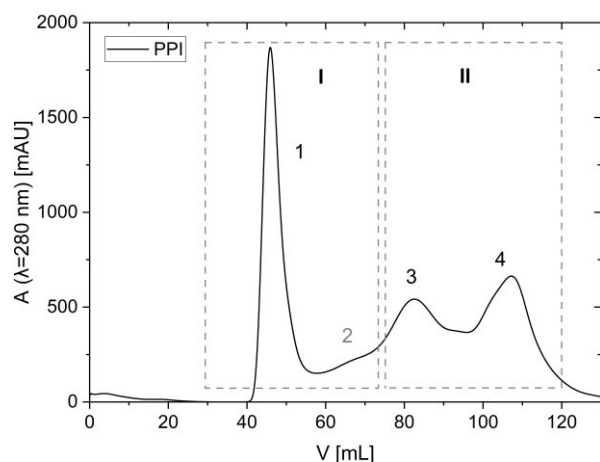


Figure SI 3 7: Chromatogram of PPI obtained from SEC. Peaks 1 and 2 are summarized in fraction I and peaks 3 and 4 are summarized in fraction II.

Table SI 3 1: MS data of PPICur t24 and PPIp of fractions I and II.

Accession	Protein	Scores PPICur II	Score PPICur I	Score PPIp I	Score PPIp II	Peptides PPICur II	Peptides PPICur I	Peptides PPIp II	Peptides PPIp I	SC (%) PPICur II	SC (%) PPICur I	SC (%) PPIp II	SC (%) PPIp I
sp P13918 VCLC	Vicilin	821.8	1761.2	4099.3	426.3	19	30	64	11	26.8	47.9	76.7	17.9
sp P15838 LEGA2	Legumin A2	706.6	943.1	3090.8		12	18	50		22.7	26.9	43.7	
sp P13919 CVCB	Convicilin (Fragment)	911.2	1670.1	2559.2	352.7	21	31	42	9	37.3	40.4	53.6	11.4
sp P02854 VCLB	Provicilin (Fragment)	864.0	1280.9	2552.3	421.8	19	24	45	9	27.6	41.5	45.4	20.7
sp P02857 LEGA	Legumin A			2507.4	588.5			40	12			44.1	21.1
sp P13915 CVCA	Convicilin	552.4	1414.3	2392.9	69.2	15	31	43	2	19.8	31.0	42.0	2.8
sp P08688 ALB2	Albumin-2	827.6	1025.3	2370.6	656.3	15	21	38	13	45.9	60.6	78.4	42.9
sp P02855 VCLA	Provicilin (Fragment)	211.5	332.6	1875.9	147.3	5	9	24	5	21.1	38.9	56.4	14.5
sp P02856 VCL1	Vicilin, 14 kDa component	215.0	388.9	1122.2	106.5	5	9	16	3	29.8	64.5	53.2	16.9
sp P14594 LEGB	Legumin B (Fragment)	350.2	437.7	1059.1	168.6	8	10	16	6	19.2	19.2	51.2	14.5
sp P14856 LOX2	Seed linoleate 9S-lipoxygenase-2	962.5	1395.9	1001.2	504.9	24	30	23	13	25.9	29.3	26.0	14.2
sp P05692 LEGJ	Legumin J	539.7	812.0	982.2	489.9	11	14	18	9	15.3	23.3	31.8	12.3
sp P09918 LOX3	Seed linoleate 9S-lipoxygenase-3	673.7	1001.2	912.3	516.6	16	23	18	16	18.1	28.5	21.4	13.1
sp P05693 LEGK	Legumin K (Fragment)	748.6	799.6	911.0	509.9	14	16	16	8	22.9	34.3	42.6	20.3
sp P34922 G3PC	Glyceraldehyde-3-phosphate dehydrogenase	115.6	171.0	346.6	65.3	3	5	7	3	7.1	18.0	22.5	6.8
sp Q41060 SBP65	Seed biotin-containing protein	57.3	63.0	310.8	77.3	2	2	6	2	2.9	2.9	14.0	5.1
sp P46257 ALF2	Fructose-bisphosphate aldolase	42.6	88.7	240.9	53.6	1	3	5	1	3.3	14.5	22.8	2.5
sp Q06930 ABR18	ABA-responsive protein			218.7				6				53.8	
sp P47922 NDK1	Nucleoside diphosphate kinase 1	90.9	170.6	193.7	94.9	3	4	4	2	20.8	26.8	34.9	20.8
sp P02867 LEC	Lectin			192.2				3				10.2	
sp P12886 ADH1	Alcohol dehydrogenase 1	96.6	229.6	176.7	45.2	3	5	3	1	11.8	17.1	14.5	2.1
sp P46258 ACT3	Actin-3	99.0	91.3	137.1	39.6	2	2	2	1	7.4	7.4	7.4	2.7
sp P30164 ACT1	Actin-1		63.9	113.5			2	2			9.0	9.0	
sp Q06931 ABR17	ABA-responsive protein			113.3				2				22.3	
sp Q41011 EF1A	Elongation factor 1-alpha	60.2	106.1	111.7	51.7	1	3	3	1	2.5	9.6	11.2	2.5
sp P25795 AL7A1	Aldehyde dehydrogenase family 7 member A1	56.7	142.3	46.4		1	4	1		2.6	8.7	2.4	
sp P25470 H2A1	Histone H2A.1		152.3				4				20.0		
sp P62788 H4	Histone H4	286.3	369.6		187.5	7	8		4	48.5	41.7		29.1

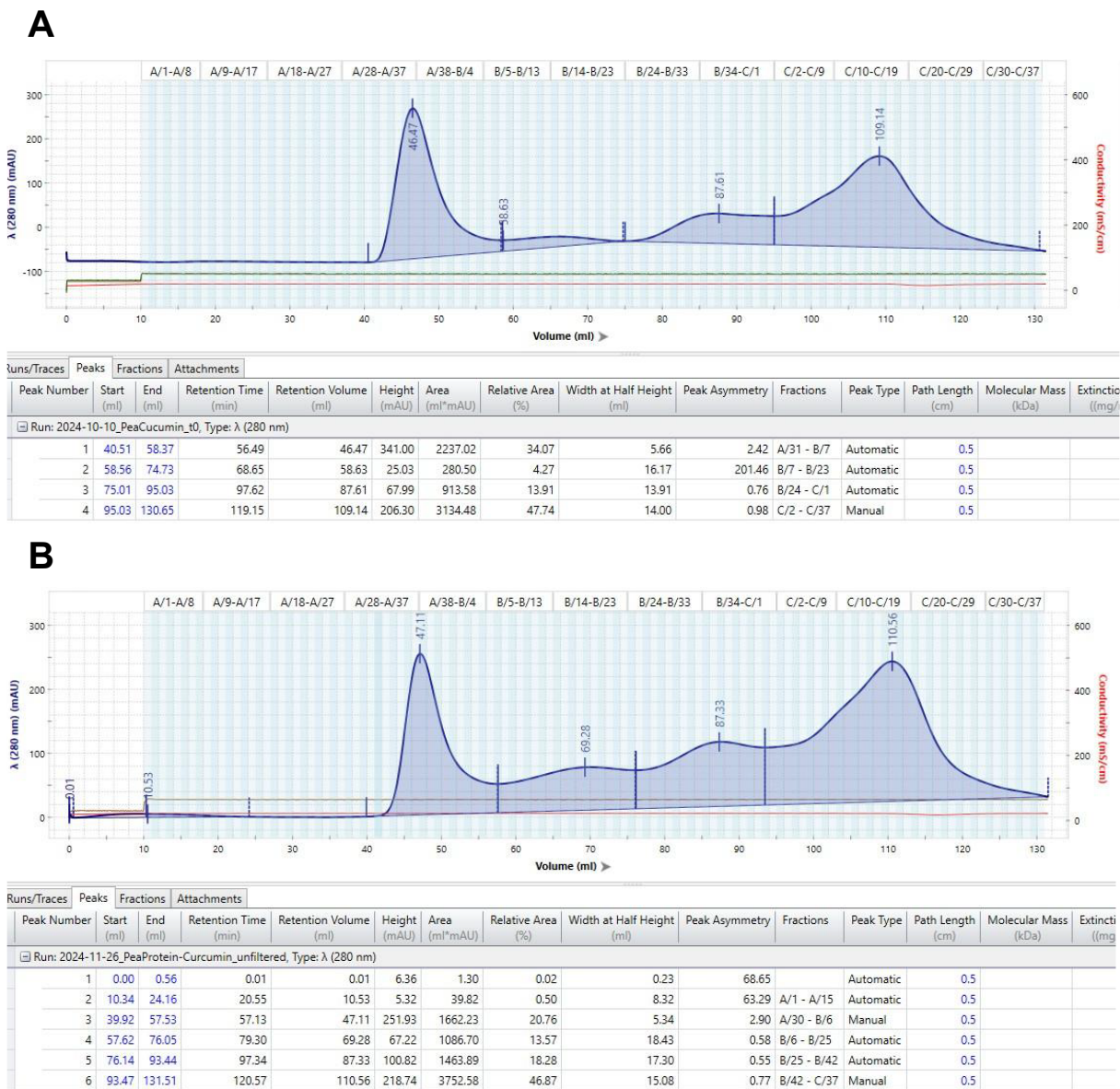


Figure SI 3 8: Areas under the chromatograms obtained from SEC of PPICur t0 (A) and PPICur t24 (B).

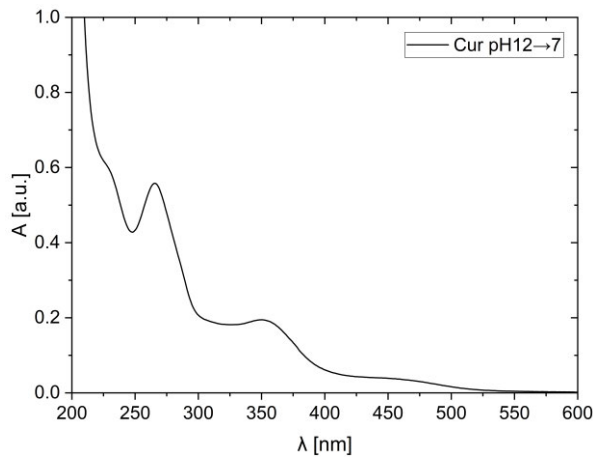


Figure SI 3 9: UV-Vis spectroscopy of pH-shifted Cur in water.

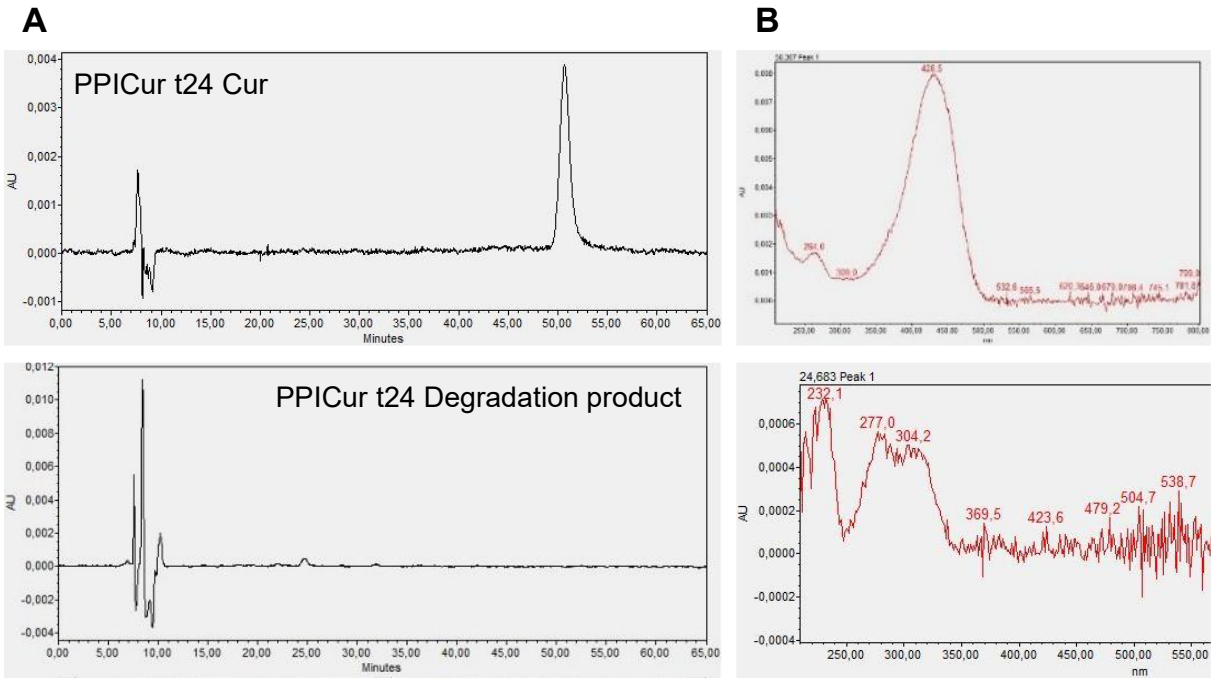


Figure SI 3 10: Chromatograms (A) and UV-Vis absorbance spectra (B) of the ethanolic extract of PPICur under constant stirring at 37 °C for 24 hours, with peaks at 49.7 min and 22.9 min with the respective mobile phases described in the experimental section. °C.

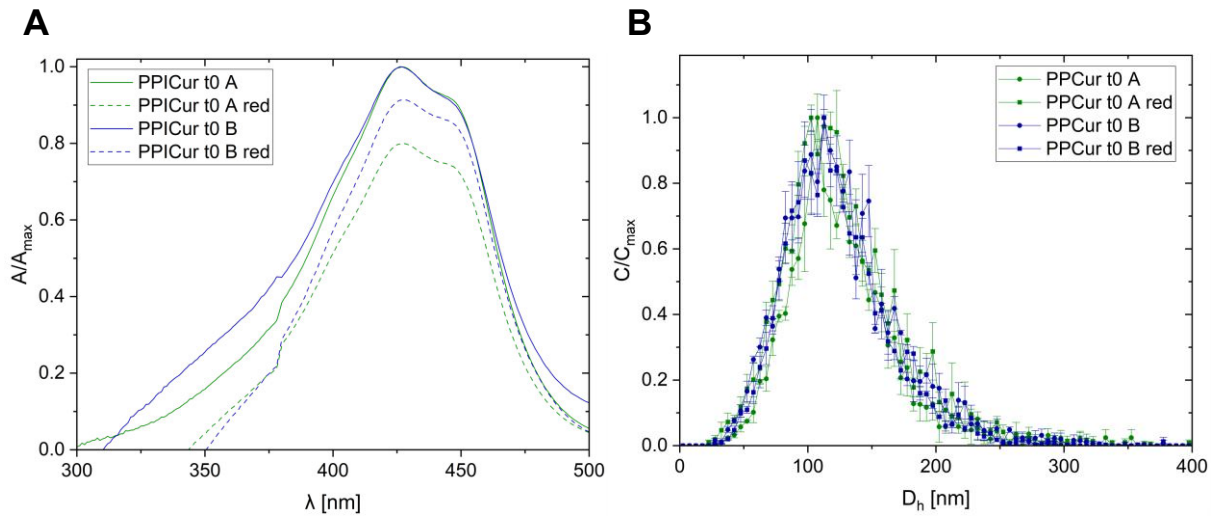


Figure SI 3 11: Check for redispersibility of PPICur t0 using UV-Vis spectroscopy (A) and NTA (B). Experiments were performed in duplicate.

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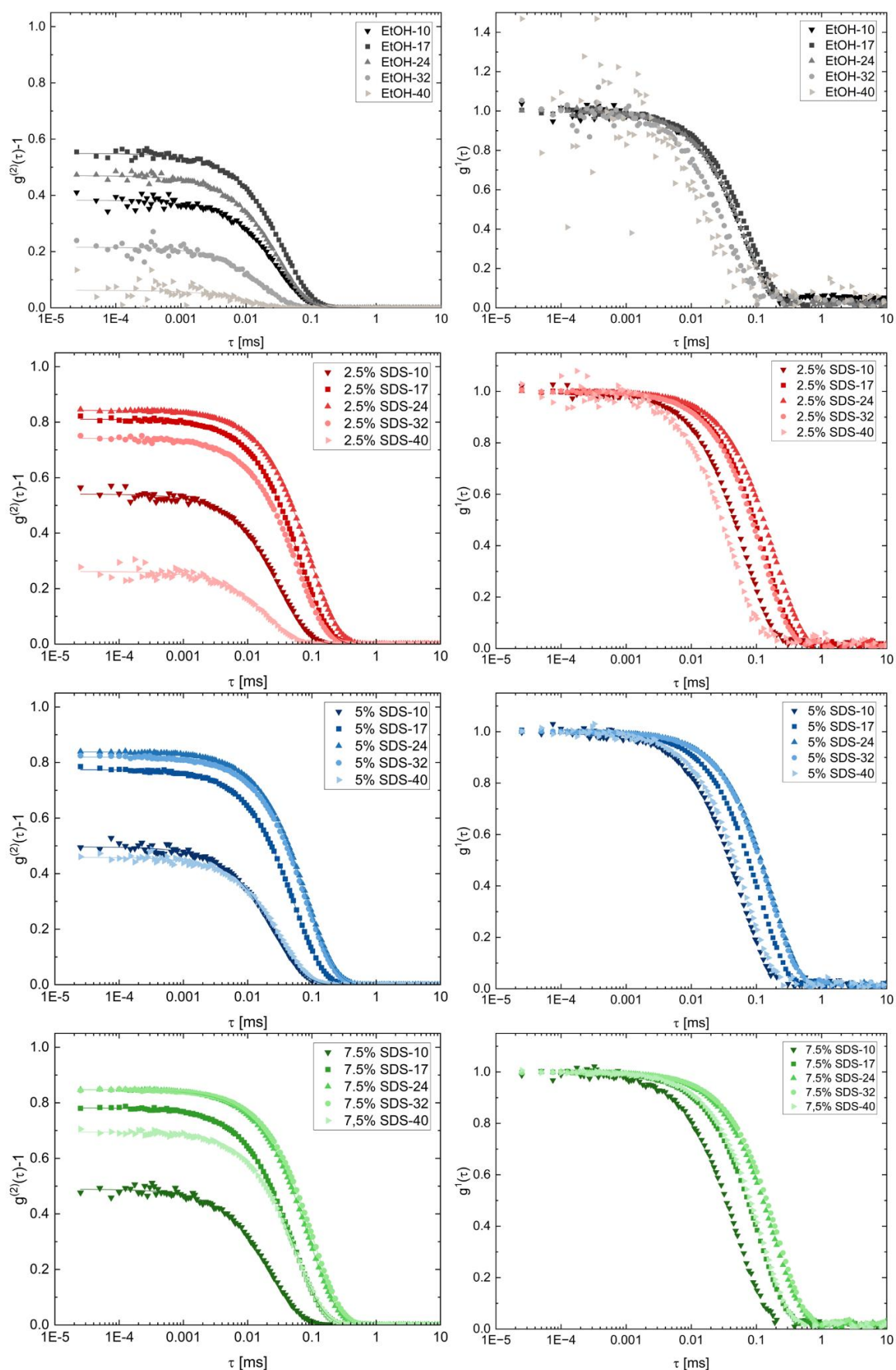


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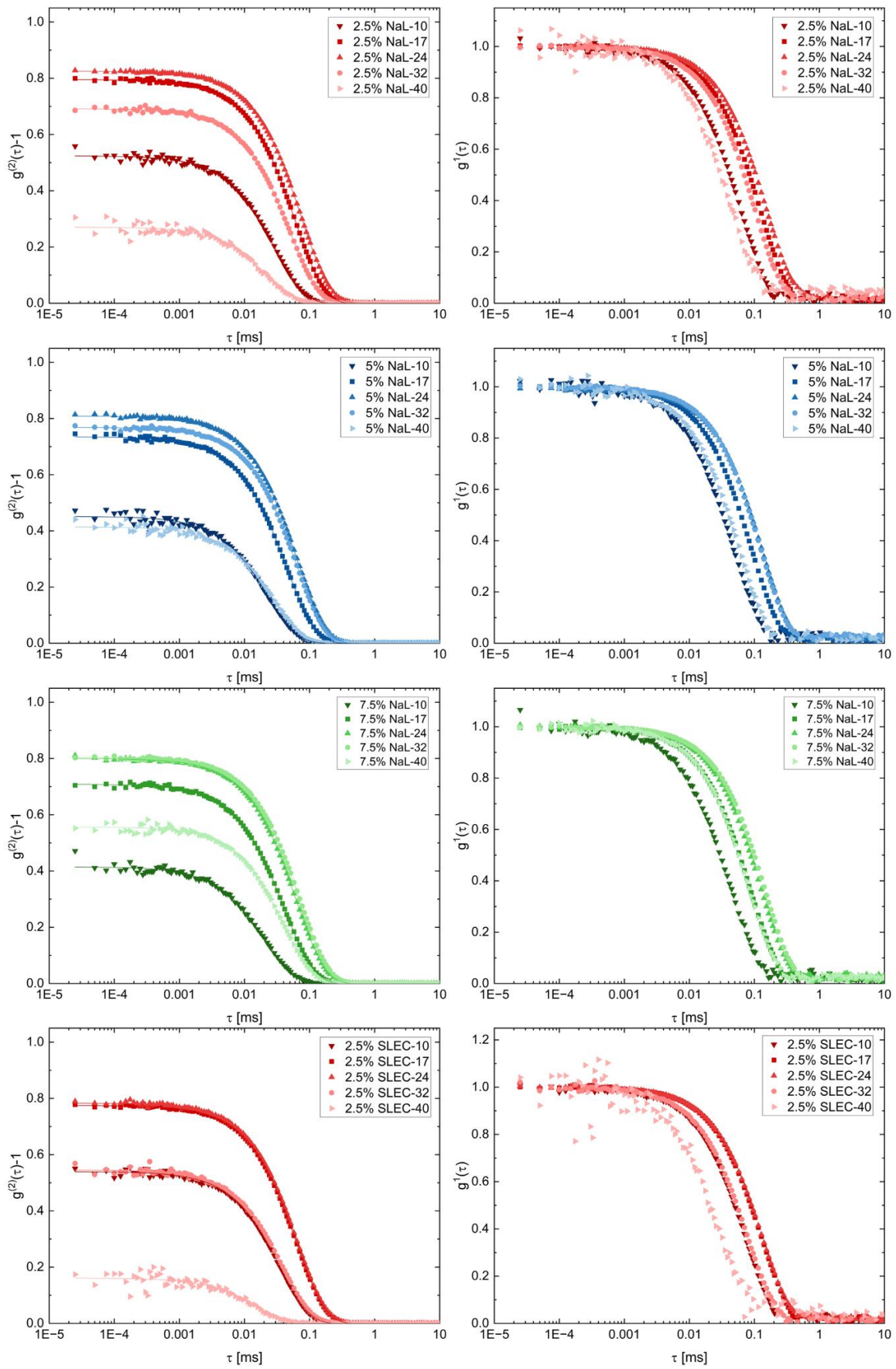


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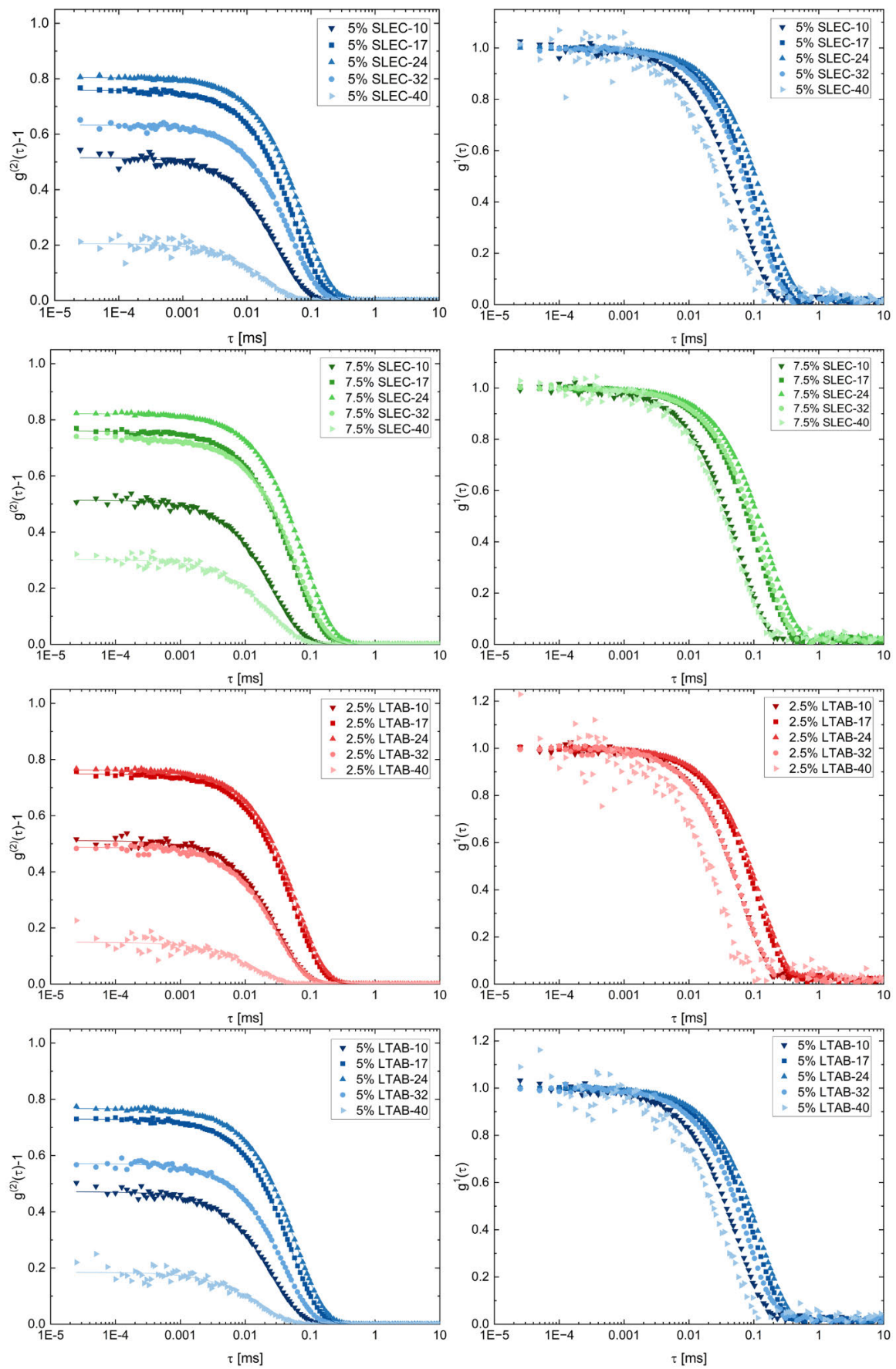


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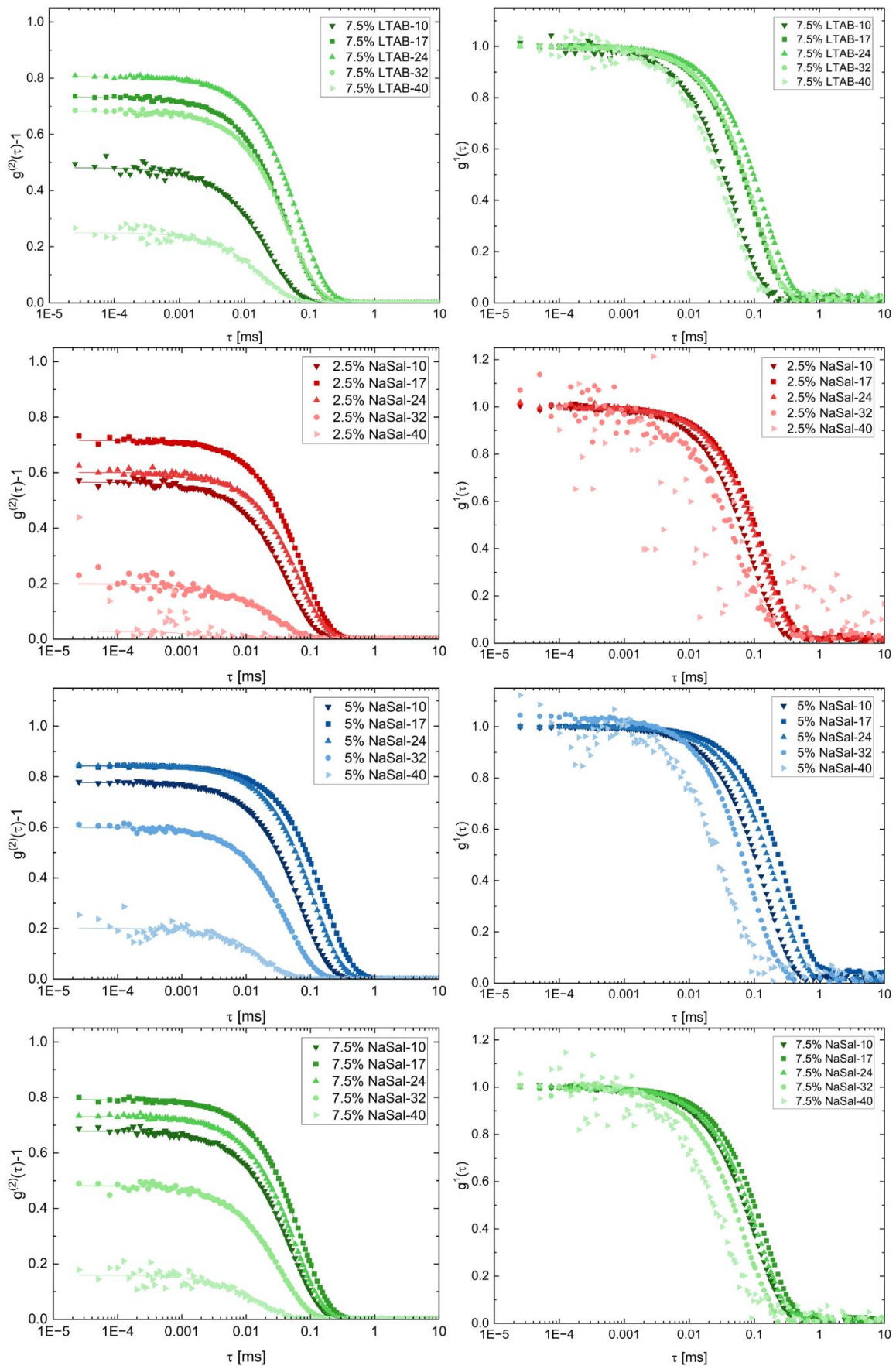


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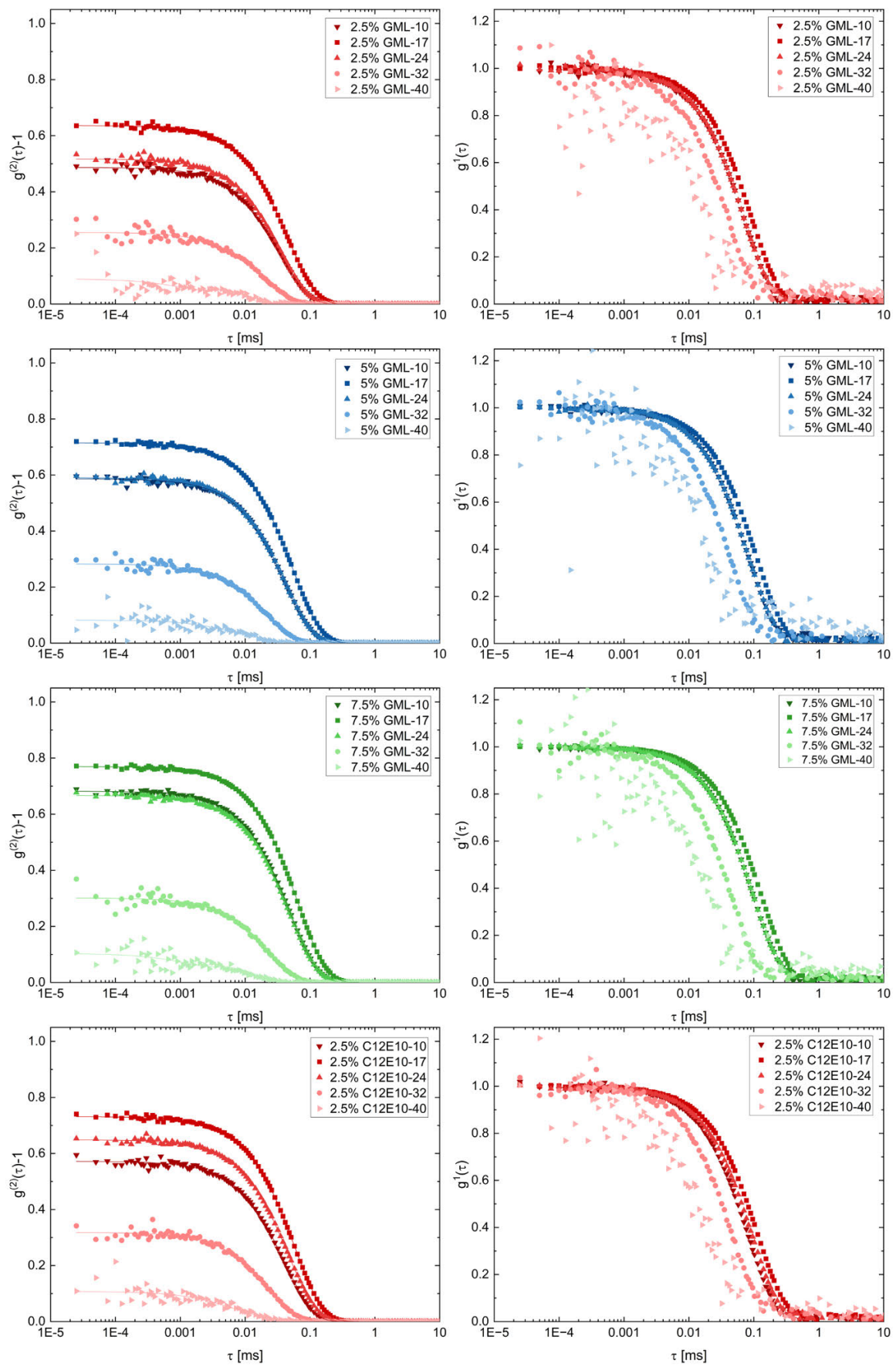


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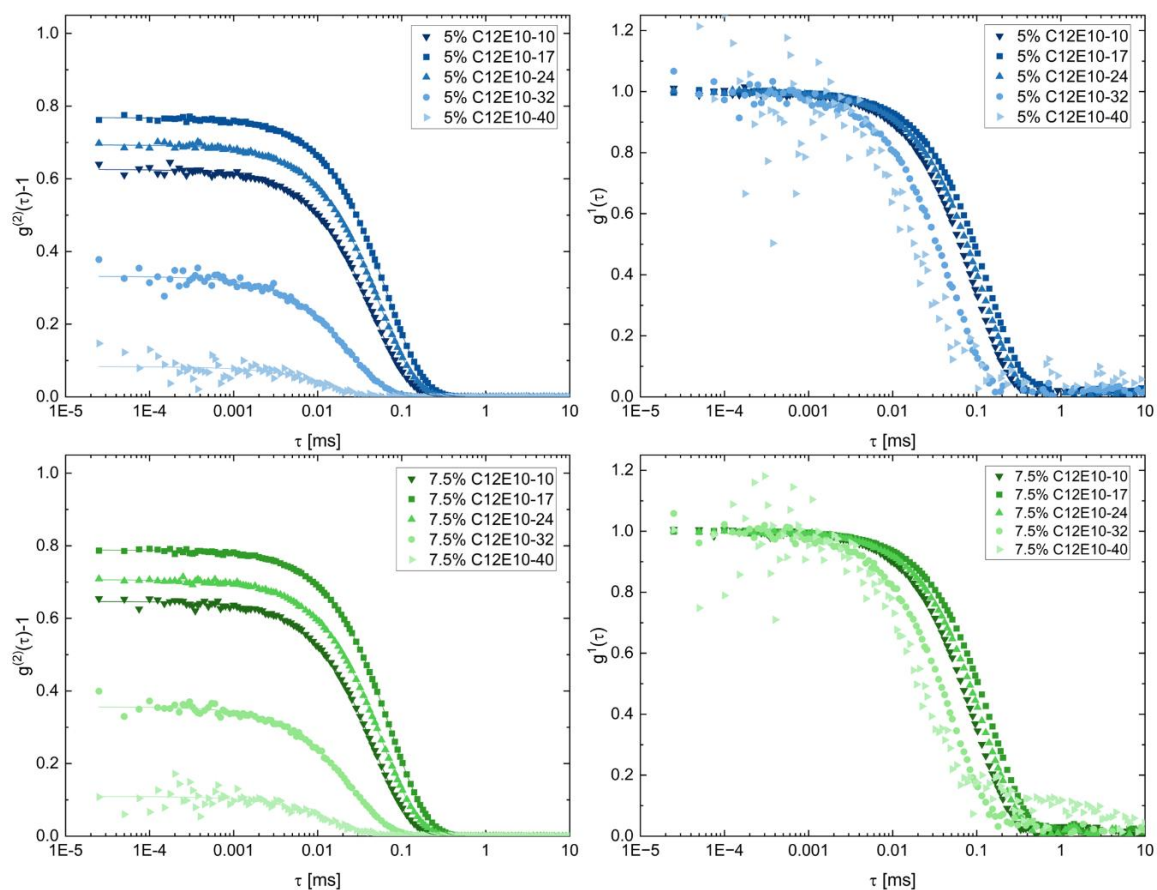


Figure SI 4 1: Intensity autocorrelation functions $g^{(2)}(\tau)-1$ and normalized field autocorrelation functions $g^{(1)}(\tau)$ of pure EtOH/Oct/Water and with the addition of 2.5-7.5% compound SDS, NaL, SLEC, LTAB, NaSal, C12E10, and GML.

Table SI 4 1: Fit parameters obtained from the second-order cumulant analysis of DLS intensity autocorrelation functions $g^{(2)}(\tau)-1$. Unreliable data due to too low autocorrelation functions are marked with ^a.

	Sample [% Oct]	β	Γ	μ_2	C
SFME	10	0.38179	15.92219	0	0.00104
	17	0.54947	12.96449	6.68288	-1.99095E-4
	24	0.47075	16.20581	23.15652	-5.23488E-4
	32	0.21573	28.52337	63.2256	1.31351E-5
	40	0.06253	43.72927	0	-2.80292E-4
SDS 2.5%	10	0.54097	15.29255	15.41076	1.57322E-5
	17	0.81196	7.51488	2.23886	-8.39866E-4
	24	0.84356	5.41583	0.95123	-6.56046E-4
	32	0.74273	8.29493	3.23161	-6.29833E-4
	40	0.26213	24.04676	39.49251	-8.70507E-5
SDS 5%	10	0.49744	19.18009	69.57933	-8.3163E-4
	17	0.77546	9.35922	6.36811	-8.48409E-4
	24	0.83967	5.96144	1.76023	-0.00118
	32	0.82108	6.18323	0.9905	-9.51253E-4
	40	0.45927	15.23934	20.94413	-3.87697E-4
SDS 7.5%	10	0.48956	21.75511	92.10503	-3.86778E-4
	17	0.78301	9.90968	12.33487	-0.00114
	24	0.84847	5.64351	2.26217	-8.19995E-4
	32	0.84975	4.85044	1.3744	-0.00246
	40	0.6959	8.80063	4.25975	-6.19559E-4
NaL 2.5%	10	0.52434	16.83588	22.37709	-5.22446E-4
	17	0.79603	8.33273	2.90727	-7.79329E-4
	24	0.82525	6.67318	1.50169	-4.82663E-5
	32	0.69144	10.1056	5.18824	-4.30843E-4
	40	0.27014	22.89274	12.50316	6.0438E-4
NaL 5%	10	0.45156	22.06331	55.41223	-1.9195E-4
	17	0.73504	11.47422	10.4189	-4.23925E-4
	24	0.81035	7.82069	3.72754	-0.00171
	32	0.7692	8.08597	2.82739	-6.89388E-4
	40	0.41542	18.14485	51.50775	-0.00112
NaL 7.5%	10	0.41483	24.57801	97.78413	-1.36502E-6
	17	0.70946	12.21916	16.22846	-7.96837E-4
	24	0.79982	8.07873	4.05417	-5.01614E-4
	32	0.8042	7.05823	2.27879	-0.0012
	40	0.55643	12.3107	8.25007	1.85479E-4
SLEC 2.5%	10	0.54035	14.18693	12.58969	-8.5502E-4
	17	0.77589	7.95908	2.92132	-0.00107
	24	0.78379	7.80806	2.986	-4.77482E-4
	32	0.54571	13.21866	13.61721	-3.25999E-4
	40	0.16048	33.62444	60.89966	3.79125E-4
SLEC 5%	10	0.51612	16.71731	31.49088	-4.3372E-4
	17	0.75933	8.89605	5.82645	-9.09691E-4
	24	0.80545	6.73439	3.07369	-9.13153E-4
	32	0.63434	10.41664	7.969	-5.31575E-4
	40	0.20587	28.617	156.78094	-1.34899E-5
SLEC 7.5%	10	0.51447	18.53075	55.32423	-7.07305E-4
	17	0.76104	9.04487	6.96248	-7.32802E-4
	24	0.82341	6.21359	3.04011	-0.00129
	32	0.7356	8.0541	7.76639	-0.00247
	40	0.30353	22.51268	156.56194	-6.48946E-4
LTAB 2.5%	10	0.51104	15.58992	7.59453	6.85321E-5
	17	0.74921	8.95295	2.27455	-3.90822E-4
	24	0.76335	7.9627	1.09027	1.31874E-4
	32	0.48848	15.84822	20.95917	-6.48165E-4
	40	0.15013	40.60547	566.39719	-5.63923E-4

Table SI 4 1 continued.

	Sample [% Oct]	β	Γ	μ_2	C
LTAB 5%	10	0.47175	19.7299	66.61221	-4.76102E-4
	17	0.73085	10.0002	4.99265	-6.92222E-4
	24	0.76798	8.21347	2.94214	-7.67326E-4
	32	0.57138	12.85964	10.31865	1.92413E-5
	40	0.18528	32.9634	325.09891	-6.09062E-4
LTAB 7.5%	10	0.48147	21.43886	76.40461	-5.71779E-4
	17	0.73322	10.6623	6.89885	-4.61621E-4
	24	0.80639	7.24711	2.06256	-4.76445E-4
	32	0.68305	10.01903	5.353	-4.80294E-4
	40	0.25031	26.46171	116.78618	-3.71866E-4
NaSal 2.5%	10	0.56648	11.44927	9.46191	-8.3145E-4
	17	0.71737	6.80791	1.37955	-5.7198E-4
	24	0.6016	7.77094	2.32057	-5.85818E-4
	32	0.20086	22.19908	285.022	-0.00119
	40 ^a	0.02921 ^a	153.383 ^a	0 ^a	-5.10656E-4 ^a
NaSal 5%	10	0.77868	7.00814	2.56458	-9.24452E-4
	17	0.84176	3.01645	0.15824	4.45139E-4
	24	0.8448	4.45824	0.45577	-4.80426E-4
	32	0.59977	11.62863	10.22316	-5.52059E-4
	40	0.20137	26.68392	49.57095	3.51682E-4
NaSal 7.5%	10	0.68056	10.14572	12.5264	-0.00162
	17	0.79255	6.94875	1.81745	-4.93591E-4
	24	0.73249	8.802	5.28284	-6.4428E-4
	32	0.48371	15.49689	41.45788	-0.00131
	40	0.15994	34.07889	429.88969	-1.80755E-4
C12E10 2.5%	10	0.57212	12.23141	0.38232	-1.8101E-5
	17	0.7307	8.7291	1.27678	5.99361E-4
	24	0.64869	10.81078	3.43895	-1.25403E-4
	32	0.31766	21.81787	13.17473	2.44284E-4
	40	0.10791	75.66499	4416.09771	-6.60446E-4
C12E10 5%	10	0.62608	11.0578	4.31425	-3.66347E-4
	17	0.76765	7.39525	0	6.91319E-4
	24	0.69438	9.23425	4.21005	-9.77692E-4
	32	0.33217	21.44842	58.75022	-6.64502E-4
	40	0.08313	36.92954	0	-1.20566E-4
C12E10 7.5%	10	0.64649	10.51577	1.09211	3.09112E-4
	17	0.78827	6.69539	0.50546	1.53668E-4
	24	0.70672	8.55749	2.60537	-2.81698E-4
	32	0.35641	20.51986	82.31872	-1.76245E-4
	40	0.10705	33.63121	0	0.00239
GML 2.5%	10	0.48786	14.57872	14.13818	-8.11249E-4
	17	0.63667	10.90746	1.28119	-3.58399E-4
	24	0.51692	14.49168	4.85787	9.79473E-5
	32	0.25596	26.96709	124.91982	-6.81742E-4
	40 ^a	0.08808 ^a	265.84588 ^a	139551.35837 ^a	0.00146 ^a
GML 5%	10	0.58585	12.00113	0	1.94909E-4
	17	0.71481	9.16746	1.45549	-6.48242E-5
	24	0.59033	12.24176	3.32081	-1.56909E-4
	32	0.28284	24.06504	46.93145	-2.79071E-4
	40 ^a	0.08197 ^a	63.36195 ^a	2371.12202 ^a	4.98001E-5 ^a
GML 7.5%	10	0.68175	10.18868	0.85019	1.56312E-4
	17	0.76965	7.64266	0.42335	7.6465E-5
	24	0.6667	10.33228	2.97367	-1.23887E-4
	32	0.30166	22.57284	27.70159	-4.81056E-4
	40 ^a	0.10097 ^a	228.53686 ^a	99561.81893 ^a	0.0023 ^a

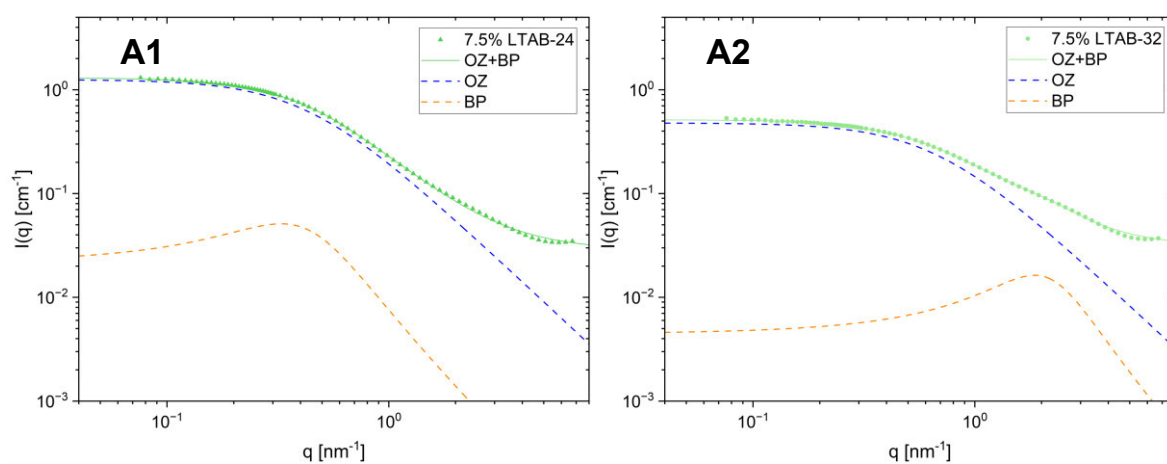


Figure SI 4 2: Visualization of the BP contribution (orange dashed line) in SAXS profiles without a clearly visible peak.

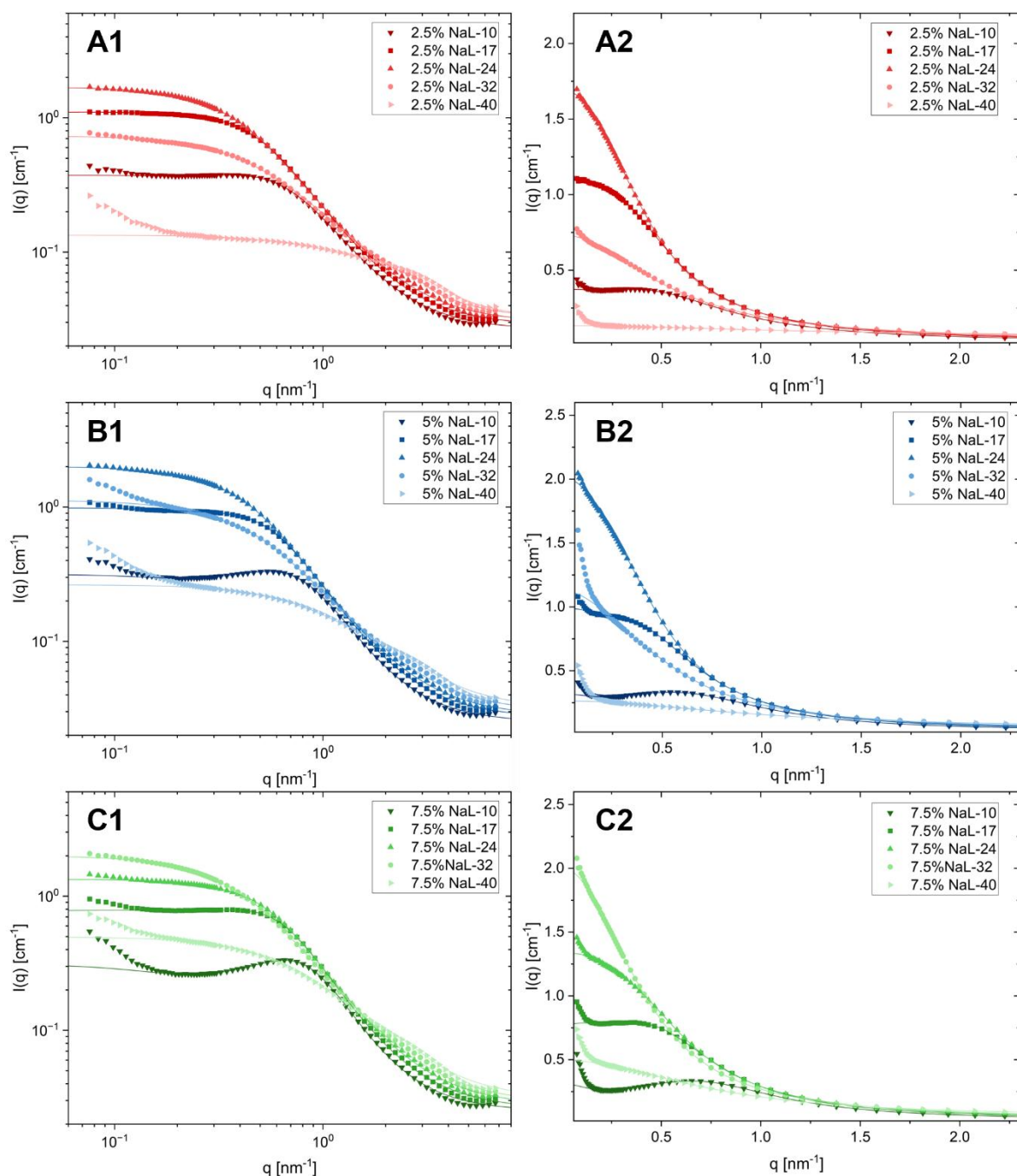


Figure SI 4 3: Scattering profiles of EtOH+NaL/Oct/Water with varying concentrations of Oct (10-40%) in log-log scale (A1-C1) and lin-lin scale (A2-C2). Symbols are SAXS data and solid lines are OZ+BP-fitted curves.

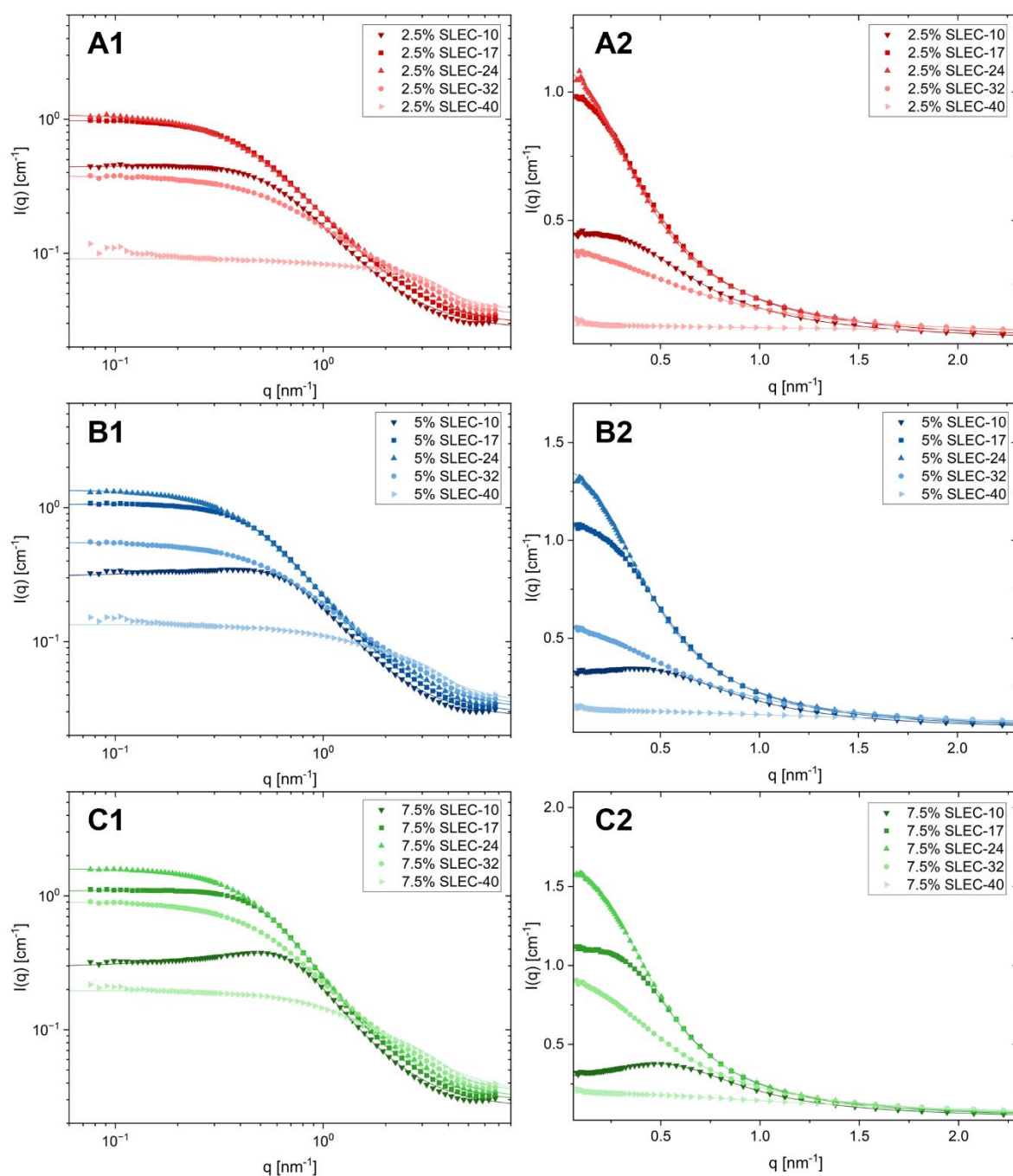


Figure SI 4 4: Scattering profiles of EtOH+SLEC/Oct/Water with varying concentrations of Oct (10-40%) in log-log scale (A1-C1) and lin-lin scale (A2-C2). Symbols are SAXS data and solid lines are OZ+BP-fitted curves.

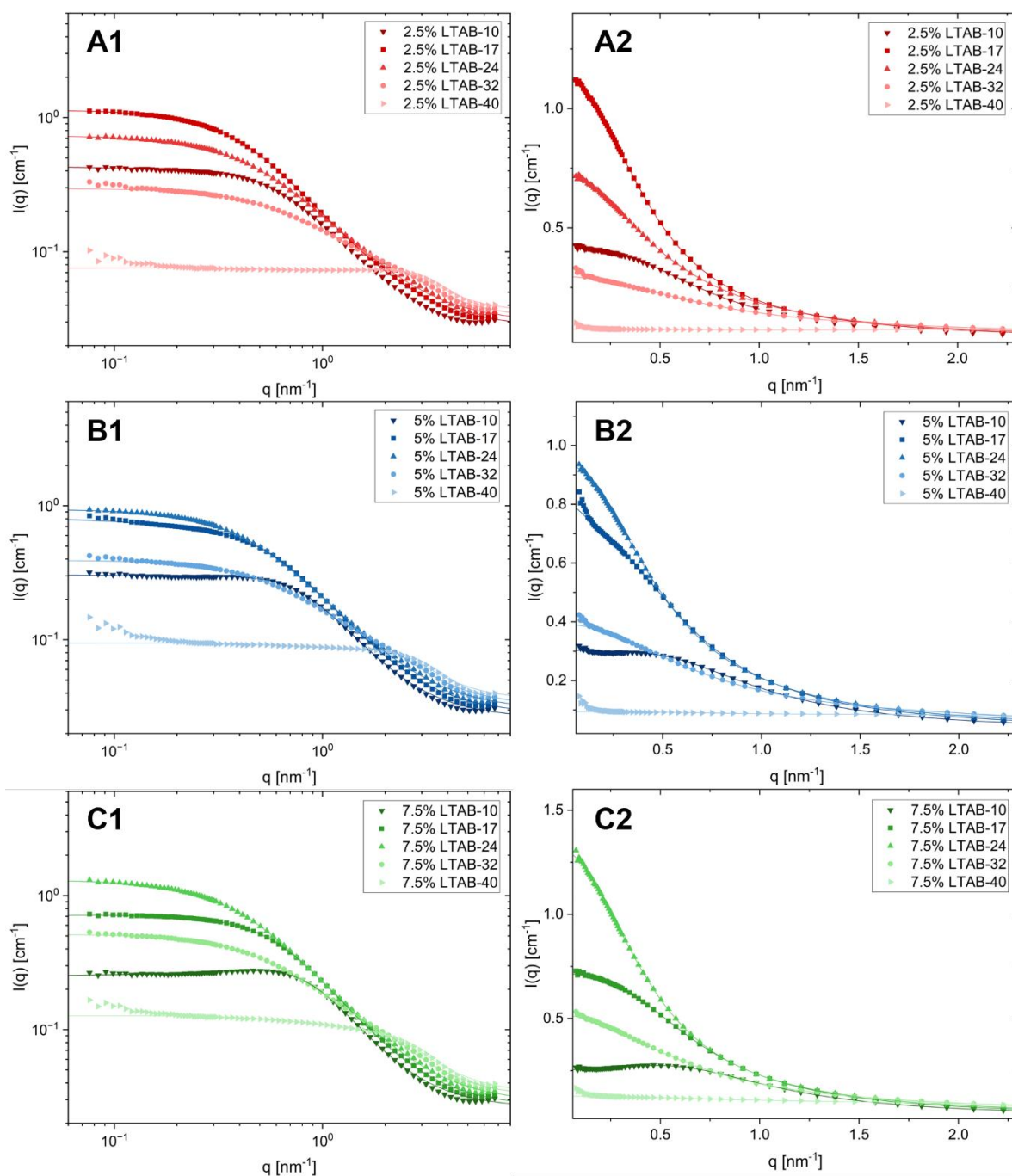


Figure SI 4 5: Scattering profiles of EtOH+LTAB/Oct/Water with varying concentrations of Oct (10-40%) in log-log scale (A1-C1) and lin-lin scale (A2-C2). Symbols are SAXS data and solid lines are OZ+BP-fitted curves.

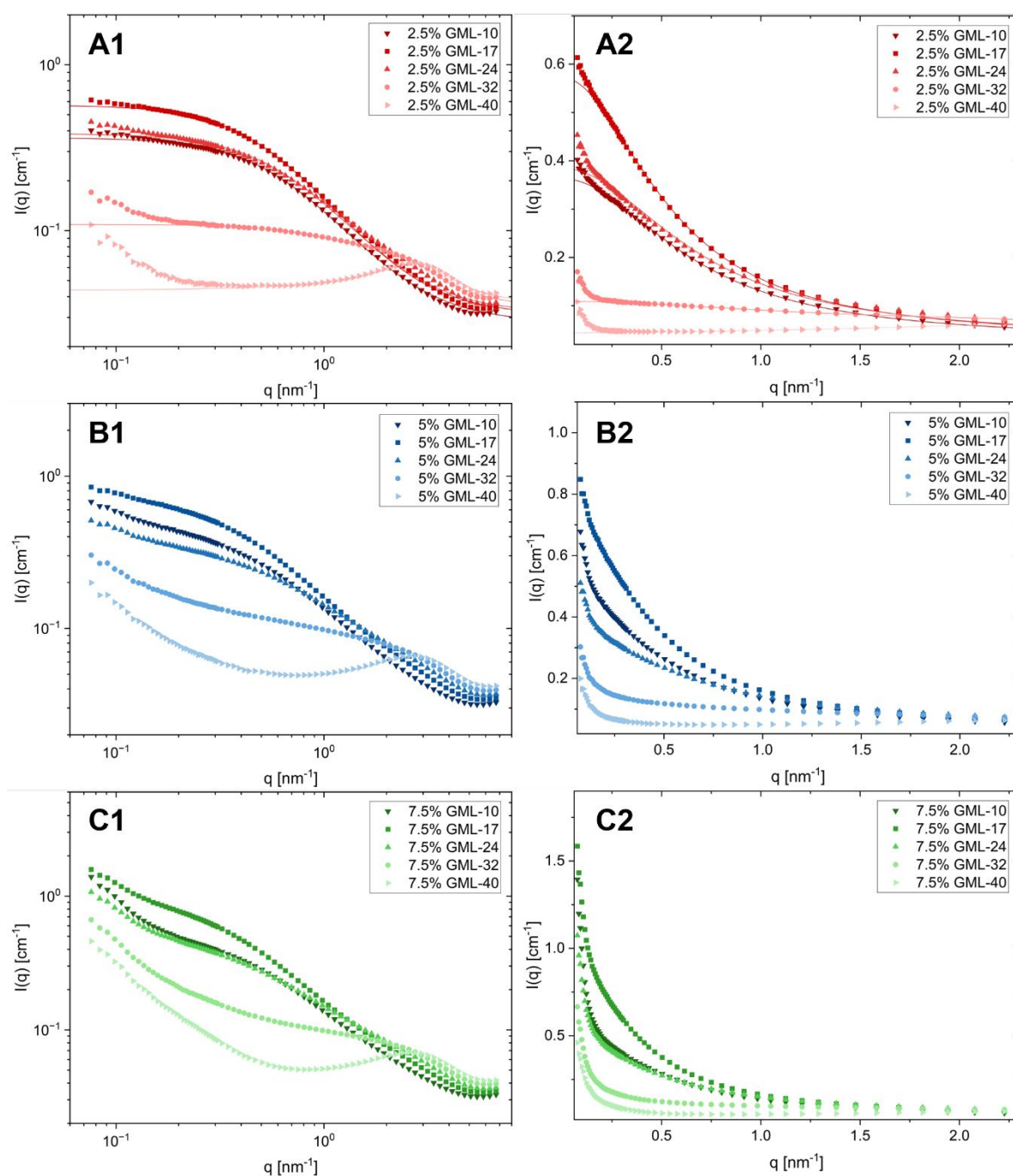


Figure SI 4 6: Scattering profiles of EtOH+GML/Oct/Water with varying concentrations of Oct (10-40%) in log-log scale (**A1-C1**) and lin-lin scale (**A2-C2**). Symbols are SAXS data and solid lines are OZ+BP-fitted curves.

Supplementary Information Chapter 4

Table SI 4 2: Fit parameters obtained from a combined Ornstein-Zernike (OZ) and broad peak (BP) fit model to the SAXS data. Parameters m and n were fixed to $m = 2$ and $n = 1$. The background was fixed (marked in grey). Fitted parameters are the OZ forward scattering $I_{OZ}(0)$, the BP intensity $I_{BP}(q_p)$, the correlation length of the OZ contribution ξ_{OZ} , the correlation length of the BP contribution ξ_{BP} , and the peak position q_p . Unreliable data due to varying OZ contributions at low q are marked with ^a.

	Sample [% Oct]	$I_{OZ}(0)$ [cm^{-1}]	$I_{BP}(q_p)$ [cm^{-1}]	background	ξ_{OZ} [nm]	ξ_{BP} [nm]	q_p [nm^{-1}]
SFME	10	0.178	0.05314	0.0285	1.55813	1.01357	0.4761
	17	0.39183	0.00646	0.0307	1.55547	1.09121	1.23475
	24	0.28238	0.01174	0.0325	1.35861	0.76716	1.58475
	32	0.06674	0.02152	0.035	0.84869	0.64459	1.96845
	40	0	0.02084	0.041	0	0.94924	2.90001
C12E10 2.5%	10	0.34091	0.04816	0.028	1.84586	1.202	0.43043
	17	0.78388	0.01228	0.03	2.26733	0.9885	0.94434
	24	0.43991	0.01599	0.032	1.70492	0.7509	1.26364
	32	0.1104	0.01863	0.034	0.88017	0.70264	1.90226
	40	0.00918	0.02615	0.038	0.96107	0.65469	2.50282
C12E10 5%	10	0.37553	0.04494	0.0275	1.87531	1.16232	0.49529
	17	0.70711	0.03155	0.03	2.23162	0.97751	0.53551
	24	0.44679	0.02782	0.032	1.769	0.74911	0.83731
	32	0.13262	0.021	0.034	0.94007	0.71613	1.71167
	40	0.01408	0.02884	0.038	0.96519	0.64524	2.32253
C12E10 7.5%	10	0.30282	0.07618	0.028	1.9387	1.16306	0.4608
	17	0.92759	0.02027	0.03	2.45071	1.02963	0.88895
	24	0.54108	0.02269	0.032	1.84892	0.83519	1.12901
	32	0.13559	0.02616	0.033	0.94305	0.68328	1.53425
	40	0.01827	0.03016	0.037	0.68007	0.64568	2.2546
GML 2.5%	10	0.3352	0	0.028	1.50806	0	0
	17	0.54098	0	0.031	1.82895	0	0
	24	0.35253	0	0.032	1.45037	0	0
	32	0.06848	0.019	0.0345	0.72571	0.71291	2.13539
	40	0	0.027	0.037	0	0.64124	2.7078
SDS 2.5%	10	0.18804	0.25303	0.027	1.75525	2.18367	0.42619
	17	0.8227	0.85669	0.029	4.30547	3.10728	0.26077
	24	0.18804	0.7091	0.031	3.79801	3.31861	0.22566
	32	0.73991	0.04645	0.0325	1.97961	4.13632	0.35379
	40	0.08869	0.01507	0.0345	0.64823	0.86227	2.39445
SDS 5%	10	0.15965	0.24392	0.02650	1.47007	2.38355	0.58251
	17	0.68377	0.82006	0.02800	3.74695	2.98981	0.34825
	24	1.44165	1.16678	0.03000	4.47390	3.40123	0.24990
	32	0.88371	0.17732	0.03200	2.15693	2.91964	0.35043
	40	0.18086	0.00712	0.03200	0.74560	1.69973	2.70739
SDS 7.5%	10 ^a	0.09474 ^a	0.32225 ^a	0.027 ^a	4.94943 ^a	2.13737 ^a	0.6294 ^a
	17	0.63424	0.84585	0.0275	3.36298	3.08653	0.40518
	24	1.72049	1.4537	0.029	4.50475	3.62545	0.27472
	32	1.41333	1.01974	0.032	4.30065	3.07756	0.24268
	40	0.45623	0.00759	0.032	1.2383	2.81805	2.85563
NaL 2.5%	10 ^a	0.12753 ^a	0.32311 ^a	0.0265 ^a	5.26265 ^a	1.83757 ^a	0.40531 ^a
	17 ^a	0.53014 ^a	0.76501 ^a	0.0285 ^a	4.58318 ^a	2.6327 ^a	0.27449 ^a
	24	1.31527	0.50103	0.03	3.3175	3.08211	0.24777
	32	0.702	0.00719	0.032	1.82586	2.53124	2.71245
	40	0.09177	0.02546	0.032	0.81233	0.61597	2.09384
NaL 5%	10 ^a	0.14292 ^a	0.29093 ^a	0.025 ^a	5.09367 ^a	1.8004 ^a	0.57193 ^a
	17 ^a	0.54733 ^a	0.69685 ^a	0.027 ^a	4.30192 ^a	2.46299 ^a	0.3664 ^a
	24	1.32824	0.92613	0.028	4.19726	2.91666	0.25184
	32	1.08143	0.05347	0.03	2.13819	3.82958	0.46456
	40	0.22867	0.01419	0.032	0.96413	0.85484	2.2006
NaL 7.5%	10 ^a	0.16642 ^a	0.29754 ^a	0.025 ^a	5.85813 ^a	1.88809 ^a	0.66527 ^a
	17 ^a	0.4494 ^a	0.60334 ^a	0.026 ^a	3.26631 ^a	2.36952 ^a	0.45338 ^a
	24	0.90748	0.64565	0.0275	3.04998	2.50656	0.34629
	32	1.77201	0.31047	0.029	2.93116	2.95754	0.27921
	40	0.46394	0.01102	0.031	1.28996	1.1198	2.33819

Table SI 4 2 continued.

	Sample [% Oct]	$I_{Oz}(0)$ [cm^{-1}]	$I_{BP}(q_p)$ [cm^{-1}]	background	ξ_{Oz} [nm]	ξ_{BP} [nm]	q_p [nm^{-1}]
SLEC 2.5%	10	0.27692	0.18962	0.027	1.77932	2.08686	0.34325
	17	0.74404	0.26887	0.029	2.38324	2.6806	0.24162
	24	1.03306	0.05293	0.0305	2.34974	4.13286	0.30828
	32	0.34319	0.01035	0.033	1.35702	1.02149	2.33593
	40	0.04826	0.02244	0.036	0.65274	0.69674	2.25692
SLEC 5%	10	0.16294	0.2021	0.027	1.50996	1.89785	0.47729
	17	0.69395	0.49601	0.0285	2.77875	2.63715	0.29935
	24	1.13323	0.29307	0.031	2.69118	2.88803	0.28096
	32	0.52121	0.00567	0.032	1.48503	2.43535	2.66514
	40	0.09483	0.01741	0.034	0.67223	0.72756	2.13999
SLEC 7.5%	10	0.16611	0.2426	0.026	1.4322	2.20111	0.55266
	17	0.64044	0.69288	0.0285	3.23278	2.71523	0.33569
	24	1.04365	0.72867	0.03	3.40152	2.78408	0.26147
	32	0.82802	0.08453	0.0325	1.92341	2.89891	0.36718
	40	0.16359	0.00522	0.032	0.6837	1.7309	2.65018
LTAB 2.5%	10 ^a	0.10076 ^a	0.34454 ^a	0.02650 ^a	6.76856 ^a	1.69369 ^a	0.24669 ^a
	17	1.01999	0.13850	0.02750	2.49379	2.99101	0.27673
	24	0.70221	0.00000	0.03000	1.87021	0.00000	0.00000
	32	0.25798	0.01653	0.03200	1.25145	0.72435	1.95472
	40	0.02934	0.03134	0.03550	0.84208	0.64430	2.19757
LTAB 5%	10 ^a	0.08872 ^a	0.25174 ^a	0.026 ^a	5.28251 ^a	1.47415 ^a	0.41775 ^a
	17 ^a	0.24197 ^a	0.58425 ^a	0.028 ^a	6.65396 ^a	1.88126 ^a	0.19134 ^a
	24	0.89132	0.03378	0.03	2.04035	3.38259	0.31785
	32	0.35539	0.01553	0.032	1.3627	0.84889	1.91793
	40	0.04713	0.03439	0.0355	0.7973	0.69861	2.01734
LTAB 7.5%	10 ^a	0.0732 ^a	0.23521 ^a	0.026 ^a	4.06904 ^a	1.47238 ^a	0.52823 ^a
	17 ^a	0.20858 ^a	0.55076 ^a	0.0265 ^a	4.44247 ^a	1.82721 ^a	0.25214 ^a
	24	1.25361	0.05112	0.0285	2.34561	3.56287	0.32681
	32	0.4793	0.01631	0.031	1.51197	0.87766	1.85926
	40	0.07773	0.0401	0.033	0.86254	0.68465	1.82679
NaSal 2.5%	10 ^a	0.32519 ^a	0.24136 ^a	0.028 ^a	2.70119 ^a	1.74948 ^a	0.22804 ^a
	17 ^a	0.91426 ^a	0.4382 ^a	0.029 ^a	3.24806 ^a	2.34778 ^a	0.13888 ^a
	24	0.9187	0.0189	0.031	2.14018	3.5642	0.3783
	32	0.27313	0.00711	0.033	1.10208	1.24641	2.49105
	40	0.04587	0.0259	0.0355	0.66926	0.64594	2.24383
NaSal 5%	10	0.72741	0.36653	0.027	3.40675	2.25281	0.17682
	17	4.54837	0	0.028	4.69653	0	0
	24	2.41814	0	0.03	3.27257	0.00E+00	0
	32	0.51849	0.00499	0.032	1.4157	1.70273	2.54824
	40	0.10132	0.02693	0.034	0.7275	0.64463	1.73513
NaSal 7.5%	10	0.40311	0.25663	0.028	2.73598	1.74012	0.27121
	17 ^a	0.65446 ^a	0.40155 ^a	0.029 ^a	2.8421 ^a	1.86165 ^a	0.21353 ^a
	24 ^a	0.43152 ^a	0.64579 ^a	0.03 ^a	3.26696 ^a	1.82114 ^a	0.1287 ^a
	32	0.3901	0.0077	0.031	1.07297	2.26939	0.78654
	40	0.09766	0.04089	0.032	0.7139	0.65488	1.60063

Table SI 4 3: Uncertainty of I_{OZ} , I_{BP} and resulting uncertainty of $I_{BP}(q_p)/(I_{BP}(q_p)+I_{OZ}(0))$, and ξ_{OZ} .

	Sample [% Oct]	$I_{OZ}(0)$ [cm^{-1}]	$I_{BP}(q_p)$ [cm^{-1}]	$I_{BP}(q_p)/(I_{BP}(q_p)+I_{OZ}(0))$	ξ_{OZ} [nm]
SDS 7.5%	10	0.13265	0.25332	0.65632044	1.24687
NaL 2.5%	10	0.14629	0.32712	0.69098667	6.35322
	17	0.6321	0.63351	0.50055704	3.38748
NaL 5%	10	0.17157	0.29418	0.63162641	6.32493
		0.12248	0.28657	0.7005745	4.06483
	17	0.54804	0.75545	0.57955949	5.69086
NaL 7.5%	10	0.59354	0.60453	0.50458654	3.20257
		0.22629	0.29933	0.56947985	7.54247
	17	0.17812	0.29798	0.62587692	6.20449
LTAB 2.5%	10	0.44955	0.6693	0.59820351	4.77458
		0.46623	0.56146	0.54633207	2.74872
LTAB 2.5%	10	0.10064	0.34209	0.77268313	6.35937
LTAB 5%	10	0.10123	0.32787	0.76408763	4.62794
		0.21939	0.10956	0.33305974	1.34456
LTAB 5%	17	0.2954	0.60147	0.67063231	8.95954
		0.1432	0.15851	0.52537205	1.40801
LTAB 7.5%	10	0.16696	0.13744	0.45151117	1.26798
		0.19651	0.57804	0.7462914	5.63188
NaSal 2.5%	10	0.18395	0.38736	0.67802069	4.23901
	17	1.12229	0.24338	0.17821289	2.97517
NaSal 7.5%	17	0.40549	0.65856	0.61891828	4.0885
		0.32088	0.74753	0.69966586	4.95865
	24	0.29186	0.79425	0.73127952	4.27575
		0.23783	0.85033	0.78143839	4.8757

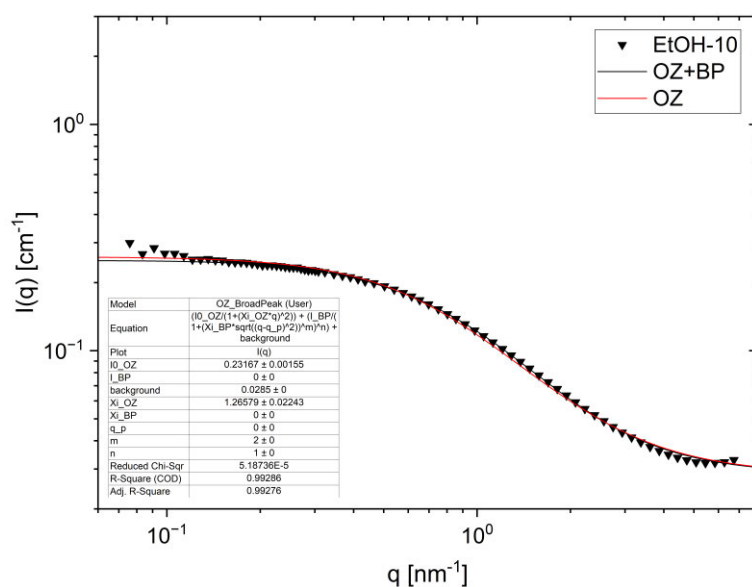


Figure SI 4 7: Fitting of EtOH-10 using the OZ model (red) and the OZ+BP model (black).

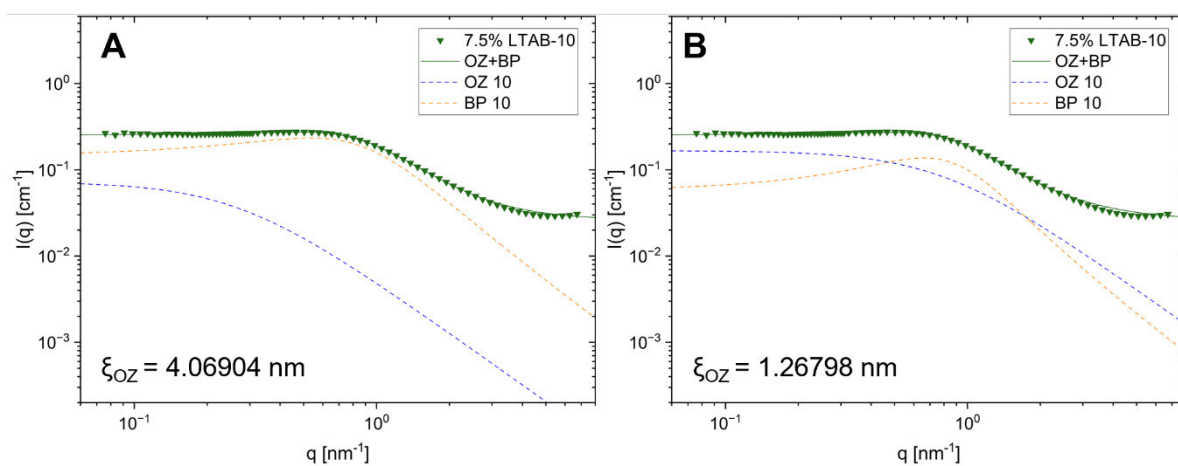


Figure SI 4 8: Varying OZ and BP contributions and resulting varying ξ_{OZ} values depending on the fitting data range.

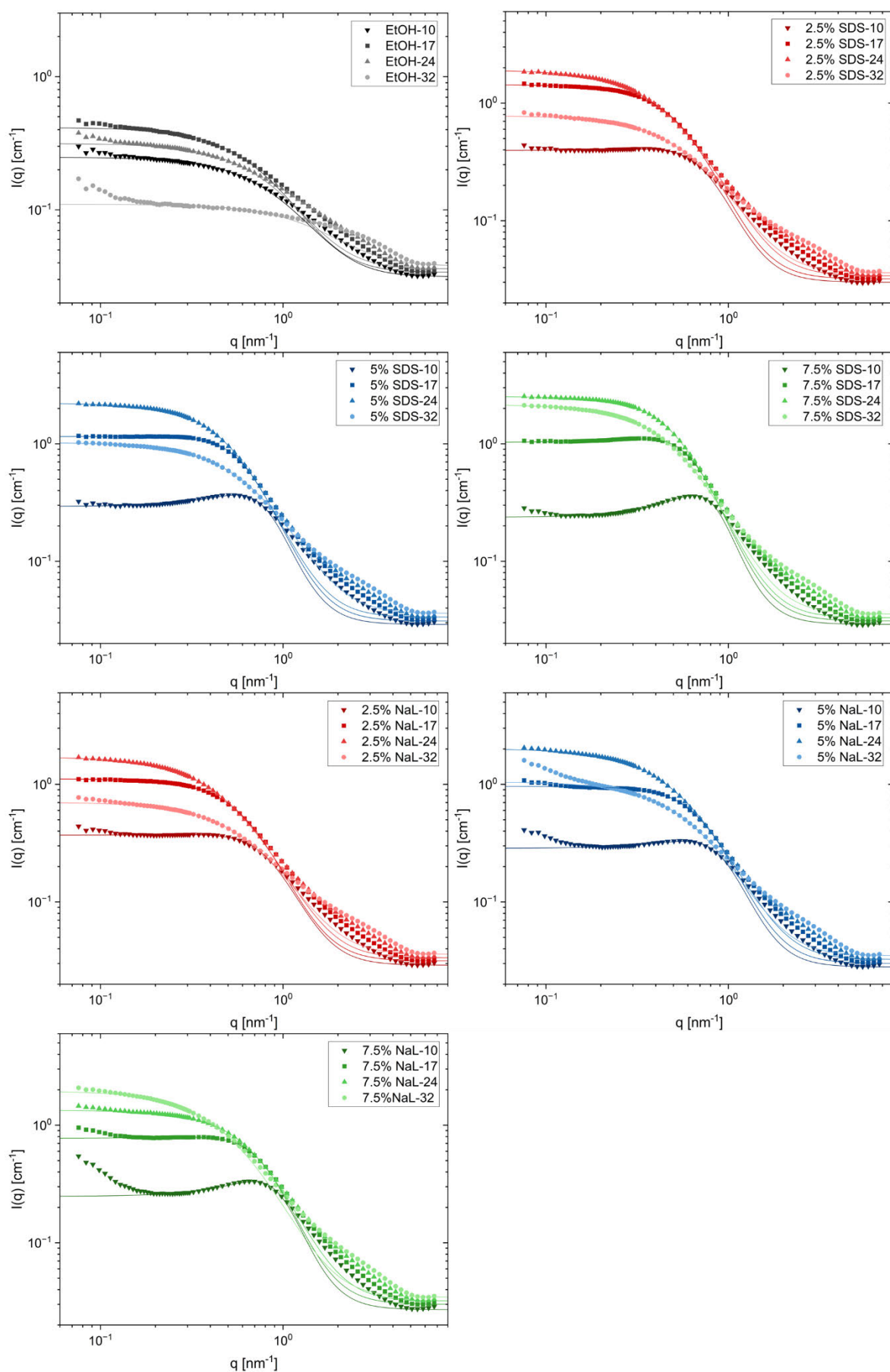


Figure continues.

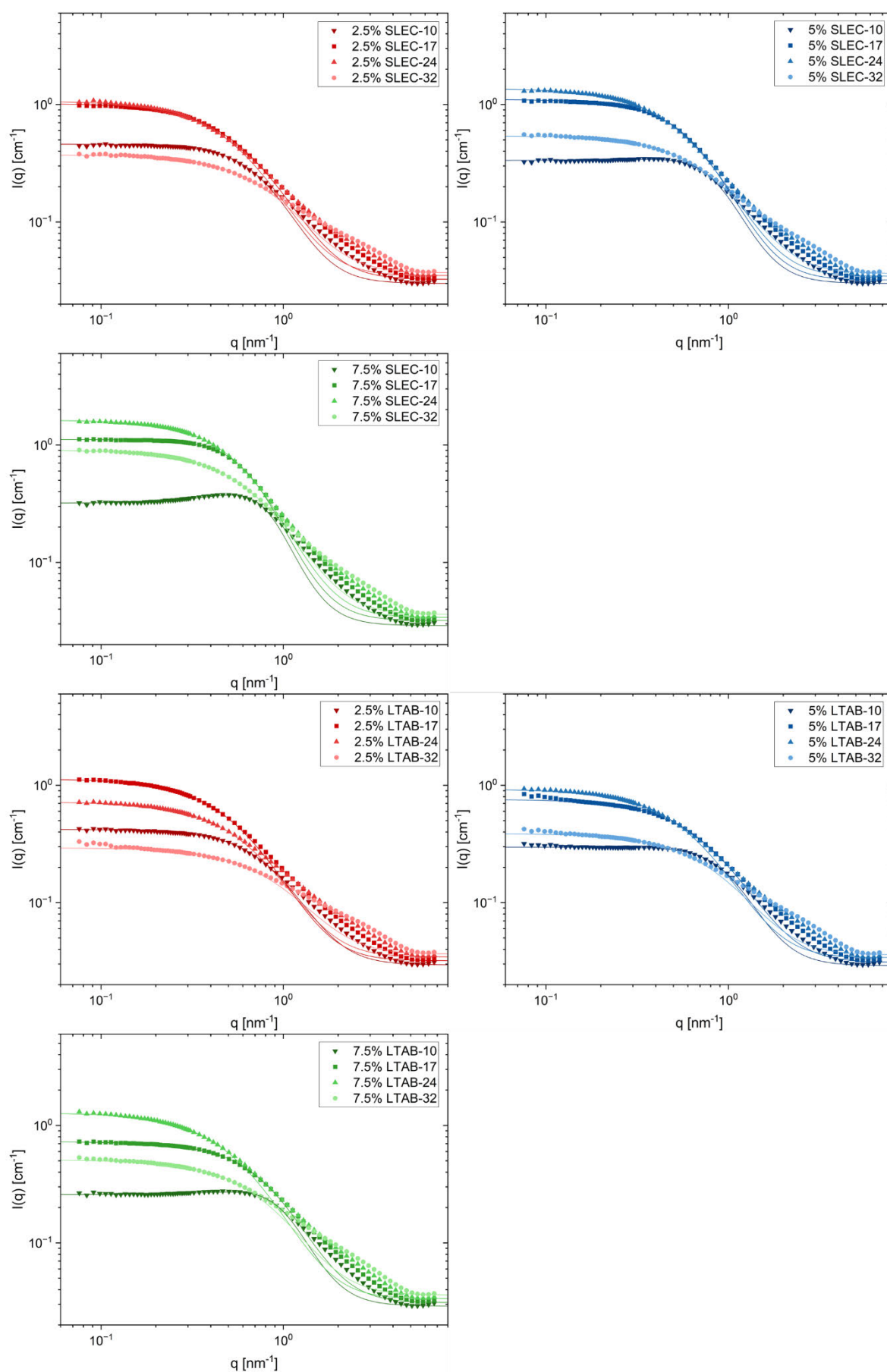


Figure continues.

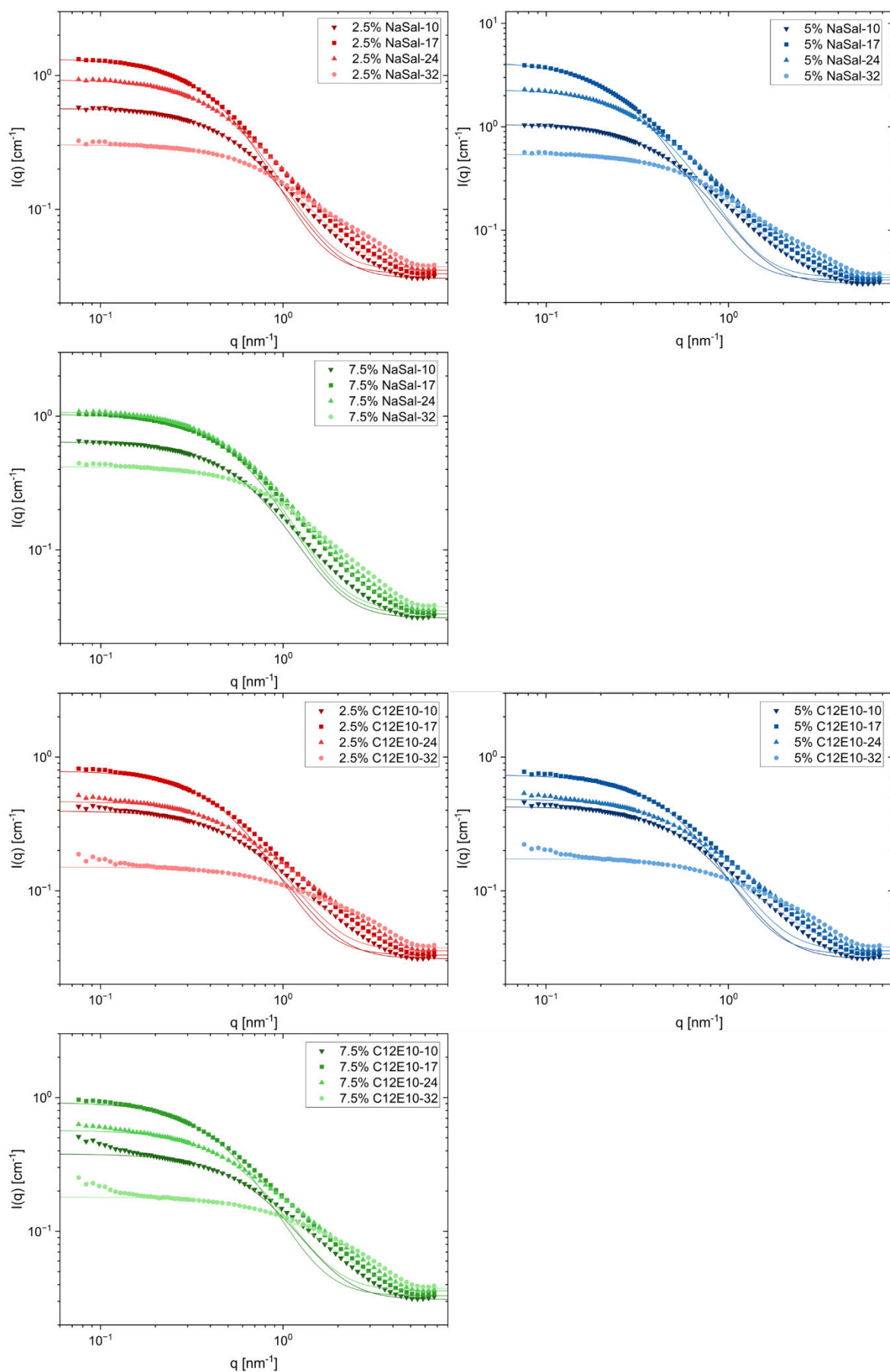


Figure SI 4 9: Scattering profiles of EtOH/Oct/Water and with the addition of 2.5-7.5% SDS, NaL, SLEC, LTAB, and C12E10. Symbols are SAXS data and solid lines are TS-fitted curves.

Table SI 4 4: Fit parameters obtained from a modified Teubner-Strey (TS) fit model to the SAXS data. Scale was set to 1 and scattering length densities were calculated and fixed at $SLD_E = 7.559 \times 10^{-4} \text{ nm}^{-2}$, $SLD_W = 9.392 \times 10^{-4} \text{ nm}^{-2}$, and $SLD_O = 8.003 \times 10^{-4} \text{ nm}^{-2}$. Volume fractions of EtOH, water, and Oct, $\phi_{E/W/O}$, were calculated and fixed for every sample (marked in gray). Fitted parameters are the background, correlation length ξ , periodicity d , and fraction of EtOH in pseudo-phase A $x_E(A)$.

	Sample [% Oct]	background	ξ [nm]	d [nm]	$x_E(A)$	ϕ_W	ϕ_E	ϕ_O
SFME	10	0.0315	0.78355	8.91137E10	0.62655	0.449	0.444	0.107
	17	0.034	0.98009	8.54231E14	0.6098	0.444	0.376	0.180
	24	0.036	0.86151	3.58059E15	0.49016	0.438	0.312	0.251
	32	0.038	0.45018	3.14952E12	0.15938	0.431	0.240	0.329
C12E10 2.5%	10	0.031	1.0096	2.1271E14	0.68641	0.440	0.454	0.107
	17	0.033	1.4131	6.50622E11	0.76923	0.433	0.387	0.179
	24	0.0355	1.0463	1.23323E10	0.57452	0.427	0.321	0.252
	32	0.037	0.51338	4.23679E10	0.1518	0.421	0.249	0.330
C12E10 5%	10	0.031	1.04454	1.07777E15	0.71364	0.439	0.453	0.108
	17	0.0335	1.35038	3.39902E13	0.73058	0.433	0.387	0.180
	24	0.0355	1.04802	4.13509E14	0.56059	0.428	0.321	0.251
	32	0.0375	0.53434	4.01466E13	0.1267	0.423	0.248	0.329
C12E10 7.5%	10	0.031	0.95461	1.05653E12	0.6712	0.439	0.453	0.108
	17	0.033	1.49176	2.1213E10	0.748	0.434	0.387	0.179
	24	0.036	1.15211	1.66679E13	0.6004	0.428	0.322	0.250
	32	0.037	0.52506	7.74566E13	0.0873	0.423	0.248	0.329
SDS 2.5%	10	0.03	2.05016	10.80887	0.83094	0.396	0.493	0.112
	17	0.032	2.13006	18.23028	0.74987	0.392	0.426	0.181
	24	0.034	1.60889	1.55708E8	0.57785	0.387	0.359	0.254
	32	0.036	1.18615	1.14083E10	0.48699	0.382	0.285	0.332
SDS 5%	10	0.029	2.39727	9.07513	0.8211	0.384	0.509	0.107
	17	0.031	2.33276	13.79124	0.70473	0.371	0.446	0.183
	24	0.0335	2.07287	21.35568	0.62203	0.366	0.379	0.254
	32	0.036	1.15841	1.90231E12	0.36672	0.361	0.305	0.334
SDS 7.5%	10	0.029	2.75711	8.43867	0.84199	0.356	0.535	0.109
	17	0.031	2.58041	12.36184	0.71333	0.351	0.466	0.183
	24	0.033	2.3309	18.612	0.608	0.346	0.398	0.255
	32	0.0355	1.68255	30.36428	0.46196	0.341	0.323	0.336
NaL 2.5%	10	0.029	1.7364	9.85439	0.76676	0.398	0.494	0.108
	17	0.0315	1.87242	15.8363	0.68128	0.393	0.426	0.181
	24	0.0335	1.52587	2.12761E9	0.56072	0.388	0.359	0.253
	32	0.036	1.11623	8.82827E17	0.44635	0.382	0.286	0.332
NaL 5%	10	0.028	1.92607	8.16236	0.74506	0.377	0.514	0.109
	17	0.03	1.80483	12.56084	0.60018	0.371	0.447	0.182
	24	0.0325	1.43617	3.83158E18	0.46176	0.366	0.379	0.254
	32	0.035	1.18854	2.82518E11	0.38692	0.363	0.304	0.333
NaL 7.5%	10	0.027	2.14973	7.544	0.74095	0.356	0.535	0.109
	17	0.03	1.97228	10.65843	0.61982	0.351	0.466	0.183
	24	0.032	1.54445	15.75582	0.4685	0.346	0.399	0.255
	32	0.0345	1.52918	1.14489E9	0.46536	0.342	0.323	0.336
SLEC 2.5%	10	0.03	1.53037	12.47256	0.76412	0.419	0.473	0.108
	17	0.0325	1.28829	4.1649E15	0.60426	0.413	0.406	0.180
	24	0.035	1.40966	1.74652E8	0.63325	0.409	0.339	0.251
	32	0.037	0.87547	1.67969E13	0.38698	0.403	0.267	0.330
SLEC 5%	10	0.03	1.76274	9.48205	0.76902	0.408	0.484	0.108
	17	0.032	1.67089	17.0172	0.6351	0.403	0.416	0.181
	24	0.0345	1.37185	1.78147E8	0.4928	0.399	0.350	0.252
	32	0.0365	0.94035	4.30008E12	0.32982	0.392	0.276	0.332
SLEC 7.5%	10	0.029	2.2347	9.20184	0.79497	0.398	0.494	0.108
	17	0.032	2.03287	13.99079	0.65117	0.392	0.425	0.183
	24	0.034	1.61197	23.20175	0.50686	0.388	0.360	0.252
	32	0.036	1.11691	6.63624E13	0.33745	0.382	0.285	0.333
LTAB 2.5%	10	0.0295	1.21626	12.62037	0.6848	0.418	0.474	0.108
	17	0.032	1.41821	5.5841E8	0.6662	0.413	0.407	0.180
	24	0.0345	1.21014	2.6664E16	0.5999	0.408	0.340	0.252
	32	0.0365	0.80266	1.0222E11	0.3911	0.402	0.267	0.331

Table SI 4 4 continued.

	Sample [% Oct]	background	ξ [nm]	d [nm]	$x_e(A)$	ϕ_W	ϕ_E	ϕ_O
LTAB 5%	10	0.029	1.36443	8.64695	0.6862	0.408	0.484	0.108
	17	0.031	1.02864	1.01141E14	0.4699	0.403	0.415	0.182
	24	0.034	1.26124	2.6432E12	0.583	0.398	0.349	0.252
	32	0.036	0.87666	1.21873E9	0.3911	0.392	0.276	0.332
LTAB 7.5%	10	0.029	1.59164	7.84294	0.7125	0.398	0.494	0.108
	17	0.031	1.23425	14.55805	0.5217	0.392	0.426	0.182
	24	0.0335	1.42288	1.12739E16	0.5951	0.387	0.360	0.253
	32	0.036	0.97624	4.51666E9	0.435	0.381	0.285	0.333
NaSal 2.5%	10	0.0305	1.12537	4.35897E8	0.72949	0.418	0.474	0.107
	17	0.033	1.61521	8.47705E16	0.73473	0.413	0.406	0.181
	24	0.035	1.32434	2.78086E8	0.59974	0.409	0.340	0.251
	32	0.037	0.72196	1.67252E13	0.25749	0.402	0.267	0.330
NaSal 5%	10	0.0305	1.55198	3.16068E9	0.80553	0.398	0.494	0.108
	17	0.033	2.79816	1.10426E11	0.85698	0.393	0.426	0.182
	24	0.035	1.99799	2.24889E19	0.73678	0.388	0.359	0.253
	32	0.037	0.91837	1.04948E13	0.32821	0.381	0.285	0.333
NaSal 7.5%	10	0.031	1.11088	2.09272E17	0.67022	0.398	0.494	0.108
	17	0.033	1.25155	1.11035E10	0.58723	0.396	0.424	0.180
	24	0.035	1.23315	4.94236E10	0.49349	0.387	0.360	0.253
	32	0.037	0.68725	2.72694E8	0.05367	0.383	0.285	0.333

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Scientific Contributions

Conference contributions:

- 04/2023 18th Zsigmondy Colloquium of the German Colloid Society, Berlin, Germany, *Supersaturation in Colloidal Systems: A comparison of Curcumin Solubility in Micellar and Plant Protein-based Systems* (poster presentation)
- 10/2023 Congress: The Future of Oil Seeds: Prospects for Plant-based Proteins? of the German Society of Fat Science (DGF), Frankfurt am Main, Germany
- 09/2024 21st IUPAC International Symposium on Solubility Phenomena and Related Equilibrium Processes (ISSP21), Novi Sad, Serbia, *Supersaturation of Curcumin in Plant-Protein-based Carrier System* (poster presentation)

Publications:

- (1) Rohr, L.; Stefan, N.; Bruckmann, A.; Touraud, D.; Dürkop, A.; Sprangers, R.; Kunz, W. Mechanistic insights into the preparation and stabilisation of supersaturated pea protein isolate-curcumin nanoparticles prepared by a pH-driven method. *Food Chem.* 2026. *Under review.*
- (2) Rohr, L.; Denk, P.; Blahnik, J.; Müller, E.; Sztucki, M.; Kunz, W. The influence of surface-active compounds on surfactant-free microemulsions. *In preparation.*

Eidesstattliche Erklärung

- (1) Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.
- (2) Die zu Beginn eines Kapitels aufgeführten Personen haben meine Arbeit in der beschriebenen Weise unentgeltlich unterstützt.
- (3) Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.
- (4) Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Ort, Datum

Lea Anna Rohr