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White proteins from green leaves in food applications

– A literature study

Anna-Lovisa Nynäs

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Abstract

This introductory paper examines the current understanding of how green leaves could be utilized as a food source, and the importance of proteins in food structures. Green leaves have long been considered as a possible protein source for sustainable food and feed production. Proteins in green leaves can be divided into a white and green protein fraction. The white protein fraction is mainly RuBisCO, or ribulose-1,5-bisfosfat-caroboxylase/oxygenase, which has been called the most abundant protein in the world, while the green fraction consists of chlorophyll related proteins. A selection of reported leaf protein extraction methods are presented in this paper. Generally the first step of the process is the extraction of green juice, followed by a removal of the green protein fraction and a concentration and purification of the white protein fraction. The functional properties of proteins as gelling agents, emulsifiers and foam stabilizers are of great importance in many food systems. One example is the interfacial properties of a protein, which control the stabilization of foams and emulsions. In this paper the role of proteins in gels, emulsions and foams are reviewed, with emphasis on foams. Some of the methods and techniques used to observe and quantify these functional properties are mentioned, including imaging techniques and surface tensiometry analyses. Some methods used to assess the properties of the food structures are also presented.

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Background

Today there is a shift in the preferred source of protein from animal to vegetal in many European countries, and the demand for protein rich vegetarian options has grown. This has been evident in recent years with a large market increase of food products based on alternative protein sources, such as oat, legumes and algae (Fernqvist and Göransson, 2017, Ridderheimsrapporten, 2015). One of the most common crops used in milk and meat replacement products is the soy bean. The main part of the soy consumed in Europe is imported. A step towards a protein independence in the EU, includes soy replacement with other locally produced protein sources (Sozer and Poutanen, 2015).

Proteins from green biomass offer an alternative to soy. The possibility of using widely available green leaf material, such as agricultural or horticultural waste streams, for production of food proteins was acknowledged already early in the 20th century (Pirie, 1942, Pirie, 1987). Fresh green leaves consists of between 1.6 and 8.2 % (wet weight) protein (van de Velde et al., 2011), and approximately 50 % of the water soluble proteins are RuBisCO, or Ribulose-1,5-bisphosphate carboxylase/oxygenase. RuBisCO catalyzes the uptake of CO_2 into photosynthesis, and is present in relatively high amounts in all photosynthetic organisms (Andersson and Backlund, 2008). In some plants up to 28 % of the total protein is RuBisCO (Evans, 1989). This high protein content in green leaves makes the leaves a promising source of food and feed proteins.

Food products are composed of several different building blocks, e.g. fat, protein and carbohydrates in various combinations. The structure of the composing building blocks are of great importance for the perception by the customer, and also for nutritional value and food stability (Aguilera and Stanley, 1999). As proteins are among the most important building blocks, properties of the proteins present in the food may contribute to highly differentiated structures. RuBisCO has been shown to have good foam stabilizing properties, which could be exploited in food applications, for example in freeze-dried foams.

In this paper some of the fundamental principles needed to understand how proteins contribute to the structure and texture of food structures are assembled. This paper also reviews some of the reported methods for green leaf protein extraction and presents an overview of some of the techniques used for characterization of the proteins and structures built from them.

The importance of food structure

Consumer perception of a food product is highly affected by the structural properties of the product. Textural sensations experienced from eating are mainly derived from the food structure, which is often also regulating the release of taste and aroma compounds (Vilgis, 2013). Bioavailability of nutrients and the digestibility of food are other aspects determined by the structure and so is the stability and shelf life of the food product (Singh et al., 2015, Aguilera and Stanley, 1999). The perceived texture of many food items function as a key to their quality, since even small alterations of the structure will be readily detected by the consumer (Szczesniak, 2002). When fruits and vegetables, for example an apple, are ripening or rotting the internal structures are degraded. Degradation alters the texture of the fruit from crispy to soft, resulting in rejection of the fruit by the consumer after just touching (Kilcast and Lewis, 1990). The sounds rising from biting and chewing the food, are also influenced by its structure, and a different auditory event than expected makes the product less acceptable (Aguilera, 2005).

Keeping textural properties unaffected when searching for healthier food options by decreasing the amounts of fat, sugar and salt, is a challenge for the food industry (Selway and Stokes, 2014). In many cases the characteristic texture of a product relies completely on fat, for example whipped cream. Another challenge is to develop products accepted by consumers with special demands. Children, especially young ones, are sensitive to new textures and their tolerance for changes is limited (Szczesniak, 2002). Food structure is also important for elderly or ill people having problems with chewing and swallowing food (Nystrom et al., 2015).

Proteins in food structures

The major compounds of food products are water, lipids, carbohydrates, and proteins (Aguilera and Stanley, 1999, Vilgis, 2013). Together these molecules form the structural elements which make up a food item. The different structural elements range in size from a few nanometers to several millimeters, and can be categorized according to their size as molecular, macromolecular, supramolecular or macroscopic elements. Examples of food components and how they are categorized according to size are presented in Table 1 and Figure 1. Many food products, for example meat, consist of hierarchically ordered elements from all size categories. Amino acids assemble into peptides, which fold into helices which form protofibrils, which together form fibers, being the backbone of the meat. The organization of, and the interactions between, the structural elements determines the structure and subsequently the texture of the food (Heertje, 1993). When dealing with food structures on a food material scale, different material structures, such as gels, foams and emulsions are commonly encountered. In the following sections these structures, and the role proteins play in them, will be described.

Table 1. Some structural elements in food according to their size. Adapted from (Aguilera and Stanley, 1999)

Molecular	Macromolecular	Supramolecular	Macroscopic
Proteins, poly-saccharides, water, lipids, sugars	Monolayers/bilayers, micelles, vesicles, liquid crystals, surfaces	Droplets, bubbles, air cells, granules, networks, fibers, crystals, glasses, cells	Suspensions, foams, gels, composites
nm	µm		mm

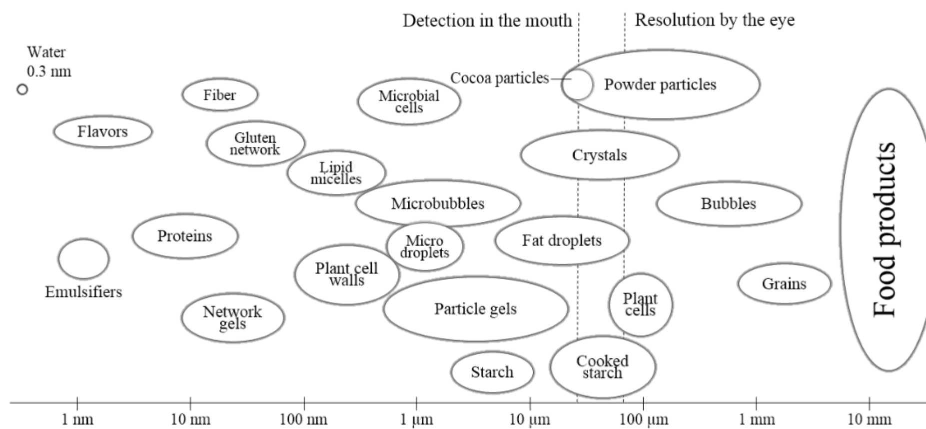

Figure 1. Some microstructural food elements and their relative sizes (approximate scales). Figure by Anna-Lovisa Nynäs after (Aguilera, 2010).

Gels

Gels are formed when polymers in a solution are interacting and form a network in which the liquid is entrapped (Aguilera and Stanley, 1999). Some examples of polymers used in food for gelling purposes are gelatin, whey proteins, starches, and pectins. In a gel the ratio of water to solid can be high, for example in gelled agar only 0.02 % is polymer, and the rest is water. Gels have also been called “soft solids”, because of the solid-like behavior, and “solid water”, because of the high ratio of water to other components. The most common gels present in food are hydrogels, in which the entrapped liquid is water. If the water is carefully removed an aerogel is formed, and in cases where the continuous phase is an oil, the gel is called an oleogel.

The structure of protein gels can roughly be divided into fine-stranded and particulate gels, as illustrated in Figure 2. The protein network in fine-stranded gels is ordered into structures in the nanometer scale, while the network in particulate gels is coarser and measured in the micrometer scale (Hermansson, 1994). Fine-stranded gels are usually transparent and particulate gels non-transparent. Gels can be a mixture between fine-stranded and particulate, but the size of aggregates in particular gels are usually

relatively uniform. However, the size of the aggregates in different gels may vary depending on protein and environmental conditions. The structure of the gel is determined by the pH and ionic strength, more repulsive forces will give a more ordered strand structure (Zayas, 1997). Around the isoelectric point of the protein, the formed gels will be less hydrated and less firm. For example the milk protein β-lactoglobulin can form both fine-stranded and aggregated particulate gels depending on pH and salt concentration (Langton and Hermansson, 1992, Langton and Hermansson, 1996)

The gelation ability of a protein depends on the amino acid composition, hydrophobicity, molecular weight and protein concentration (Zayas, 1997). In order to form the three dimensional network of a gel, the proteins need to be able to form intermolecular interactions. Stable gels are hold together by hydrogen bonds, ionic and hydrophobic interactions, van der Waals interactions and covalent disulfide bonds. Partial denaturation of the proteins through e.g. heating will expose buried chemically active groups, e.g. Sulphur groups, which may form covalent crosslinks between molecules, leading to rubbery gels. The protein concentration is essential in the gelation process. If the concentration is too low intraprotein interactions will be favored to interprotein interactions. The critical concentration needed to form a strong gel is a valuable measure when comparing different gelling agents.

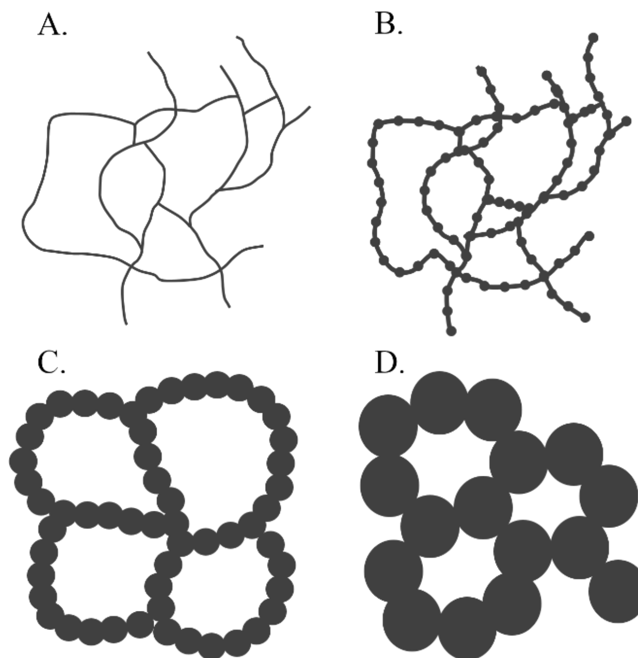

Figure 2. Different gel network structures common in biopolymer gels. A: Fine-stranded network B: Fine-stranded aggregate network, C and D: Particulate networks. Figure by Anna-Lovisa Nynäs after (Hermansson, 1994).

Emulsions

Emulsions are made by mixing two immiscible, i.e. not mixable, liquids in the presence of a stabilizing agent (McClements, 2015). A common example of an emulsion is mayonnaise, in which the two immiscible liquids are water and oil, and the stabilizing agent is lecithin, an amphiphilic compound found in egg yolk. During homogenization the stabilizer will encapsulate oil droplets, enabling the droplets to be dispersed in the continuous water phase. Due to the immiscible nature of the liquids, the stability of the emulsion depends on the strength of the interfacial film separating the two phases (Chung and McClements, 2014). Insufficient stabilization of the interfaces will lead to coalescence of the droplets (as illustrated in Figure 5, B) and eventually a complete phase separation.

The interfaces can either be stabilized by particles (Figure 3, A-C) or by amphiphilic molecules, i.e. a molecule with a clear hydrophobic and hydrophilic part (Figure 3, D) (Dickinson, 2010). Particles used as stabilizers in food are mainly protein or polysaccharide based. Pickering stabilization is one mode of action where the particles attach directly to the interface, forming a monolayer covering the droplet as pictured in Figure 3, A and B. The droplets may either be separated with a bilayer of particles or with a shared border layer (Dickinson, 2016). If the concentration of the particles in the bulk continuous phase is high, the particles may interact and form a stabilizing network barrier rather than a monolayer, as in Figure 3, C. In the case of amphiphilic molecules acting as stabilizers, for example surfactants and proteins, the molecules orient their more hydrophobic moieties towards the nonpolar phase and get adsorbed to the interfaces, which is illustrated in Figure 3, D (Lam and Nickerson, 2013). The adsorbed proteins stabilize the interfaces by forming a tight film encapsulating the droplets, as well as offering steric hindrance avoiding droplet merging. The stabilizing role of proteins in emulsions is similar to that in foams, which will be more extensively reviewed in the following section.

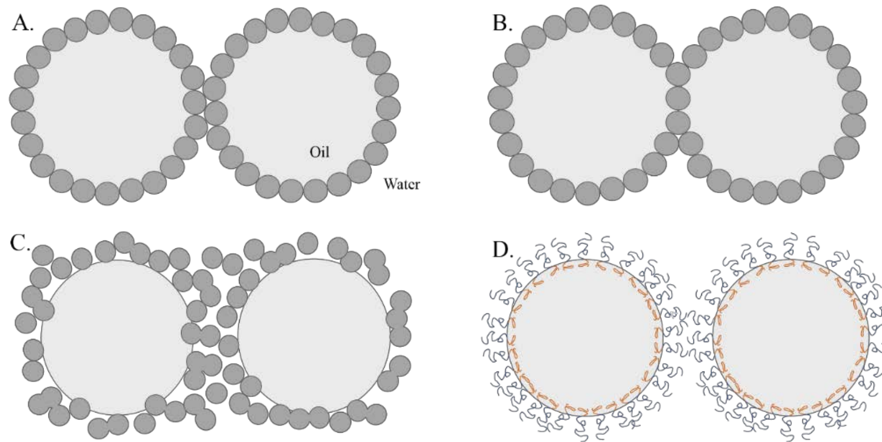

Figure 3. Stabilization of interfaces in oil-water emulsions. The same principles are valid for air bubbles in a liquid. A: A bilayer arrangement of spherical particles (e.g. protein agglomerates or starch granules) covering the surfaces of the oil droplets and B: a monolayer shared by the two droplets. C: Partially coated surfaces separated by aggregated particles. D: Steric separation by amphipathic molecules, e.g. proteins, where the hydrophilic (red) regions are adsorbed to the surface. Figure by Anna-Lovisa Nynäs after (Dickinson, 2016) (A-C) and (Lam and Nickerson, 2013) (D).

Foams

Foams are formed when air is introduced into a solution containing surface active compounds, leading to the formation of film encapsulated bubbles dispersed in a liquid continuous phase (Damodaran, 2005). At the formation of the foam most bubbles are spherical, but after time, if they are stable enough, the bubbles form polyhedral shapes with thin lamellae of liquid between (Damodaran, 1997). An example of an intersection of a foam is presented in Figure 4. The bubbles are separated by a thin liquid lamella lined with stabilizers on the gas-liquid interfaces, and the lamellae are joined in plateau borders (Fameau and Salonen, 2014).

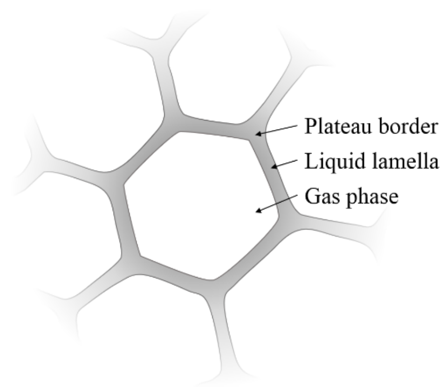

Figure 4. Illustration of a polyhedron shaped foam cell. The liquid lamellae are joined in the plateau border. Figure by Anna-Lovisa Nynäs after (Damodaran, 1996).

Similarly to emulsions, the interfaces between the bubbles need to be stabilized, otherwise the created bubbles will burst immediately. The stability of the foam depends to a great extent on the strength and flexibility of the film at the air-liquid interface, without stabilizing agents the foam will soon collapse due to its thermodynamically unstable state (Wilde, 2000). Even with good stabilization the foam will collapse over time according to one, or several, of the mechanisms of drainage, coalescence or coarsening shown in Figure 5. Due to gravity the liquid in the lamellae will drain, causing thinning of the lamellae and eventually the films collapse (Damodaran, 2005). Coalescence occurs when bubbles are in close proximity, and the film separating them ruptures causing the bubbles to merge into one bubble. Coarsening, also known as disproportionation, is driven by differences in pressure in the bubbles, resulting in shrinkage of bubbles as gas is diffused into a few larger ones. In order to reach a high stability of the foam, the processes of drainage, coalescence and coarsening need to be minimized. By an increased viscosity of the liquid phase the rate of drainage and thinning of lamellae can be minimized, and bubble shrinkage can be prevented by using surface-active compounds with good visco-elastic properties.

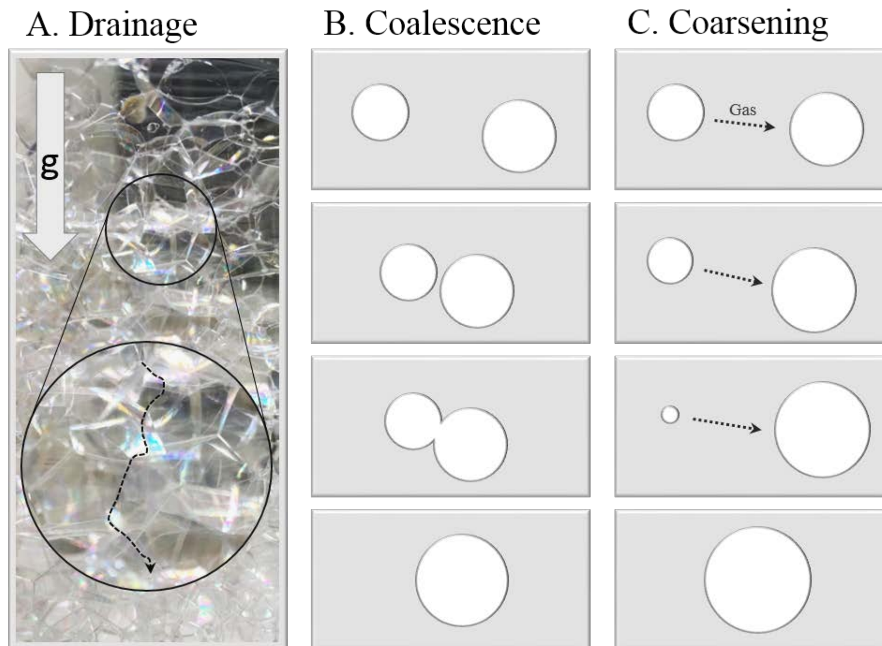

Figure 5. The three main processes of foam collapse. A: Due to gravity the liquid in the lamellae drain and thin, causing rupture of the interfacial films. B: Coalescence is the process when the interfacial films of two individual bubbles merge when the bubbles come in contact, forming one large bubble. C: Due to the Laplace pressure gas from smaller bubbles diffuse to larger bubbles, resulting in on large bubble, this process is known as coarsening or disproportionation. Figure by Anna-Lovisa Nynäs after (Fameau and Salonen, 2014).

As mentioned before, foam stability is highly dependent on the strength of the liquid films separating the bubbles. Surface active particles or molecules can be used to stabilize the interfaces, and the interactions between the particles or molecules determine the strength of the lamella. The stabilization principles for foams are generally the same as for emulsion droplets mentioned earlier and illustrated in Figure 3 (Dickinson, 2010). Proteins can be exceptionally good foam stabilizers. In nature one example of a stable protein based foams is the foam nests of some tropical frogs, which can stay intact for up to ten days (Cooper and Kennedy, 2010). Three explanations for the good foaming and foam stabilization properties of proteins are: i) the ability to strongly adsorb to the interfaces, ii) the steric stabilization of the adsorbed proteins, and iii) the structural coherence of the formed film layer (Murray, 2007). All of these functions are strongly influenced by the molecular properties of the proteins, which are mainly determined by the amino acid sequence, but also by the processing history and present conditions (Kinsella, 1981).

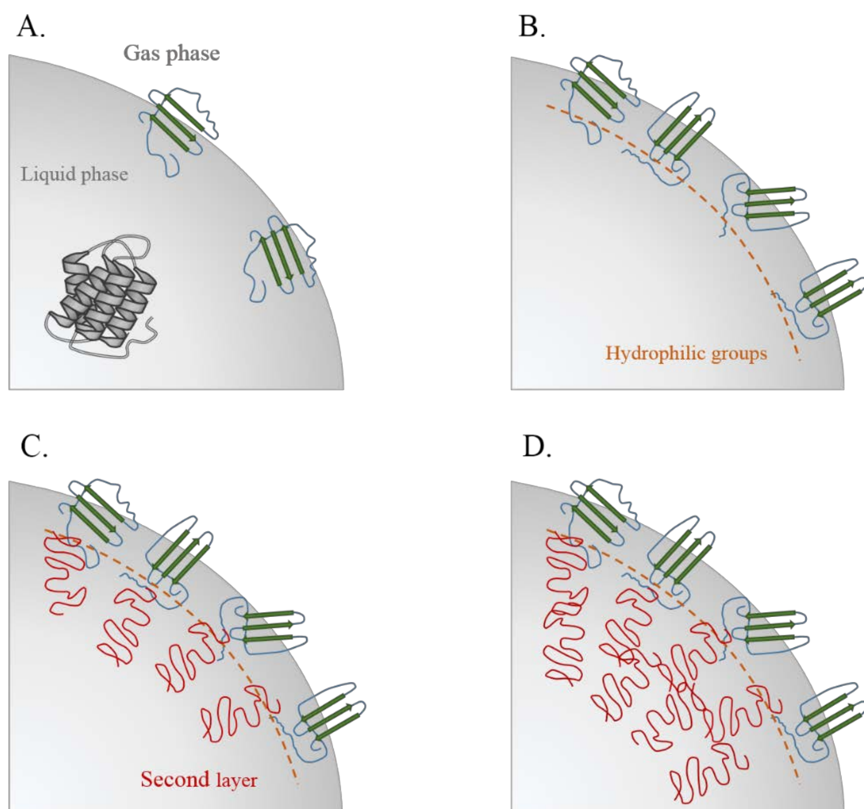

Figure 6. The steps of the adsorption process of lysozyme proteins at an air-water interface. A: Lysozyme molecules adsorb to the interphase and their conformation is changed as a result of the hydrophobic interactions with the interface B: The molecules form a monolayer and orient their hydrophilic moieties towards the water phase. C: A second layer of molecules with loose random coil structures adsorb to the first layer. D: A multilayer is formed by loosely structured molecules. Figure by Anna-Lovisa Nynäs after (Yano et al., 2008).

The process of protein adsorption at air-water interfaces is illustrated in Figure 6. It starts with the formation of a monolayer at the interface, and the rate of the adsorption is directly linked to the protein bulk concentration (Yano et al., 2008). When adsorbed, the protein can adapt to the environment, and in the case of lysozyme, the conformation of the protein changes into a flat structure at the interface. When the monolayer formation is nearly complete the proteins form connections, and the hydrophilic groups of the proteins orient towards the water phase. As the monolayer is completed, a second layer is formed by the adsorption of more loosely packed proteins in loop-like structures.

The foaming properties of a protein are dependent on several different factors, one of them being the rate at which the proteins are adsorbing to the air-water interface and the strength of their interactions. The bulk concentration of the protein and the adsorption rate is linked, and the optimal concentration varies depending on the molecular attributes of the protein (Vani and Zayas, 1995). Low concentrations allow the proteins to adsorb to the air-water interface without interactions, while more extensive folding and looping are needed at higher concentrations (Vani and Zayas, 1995, Kinsella, 1981). The unfolding of proteins at air-water interfaces only occurs if the kinetics of the adsorption and the unfolding are similar, or if the rate of adsorption is slower. In cases where the unfolding process is slower compared to the adsorption, there is not enough time for the proteins to adapt to the environment and the interfacial film will soon rupture (Wierenga et al., 2006).

The sensation perception of structure

Eating is a complex process, and the structures in the food, in sizes from molecular to macroscopic, are important for the consumer's perception. All senses, including previous experiences, are together shaping the pleasure or disgust that arise from eating a food product (Szczesniak, 2002). The full "*Gestalt*" of a food product, is the sum of all the sensorial stimulus obtained from the food. Since the sensory perception is a combination of both the psychological aspects of the consumer, and the physical and chemical aspects of the product, such perception is a challenge to understand, characterize and predict (Booth, 2005). This means that each consumer will perceive the food differently, and the individual perception have a connection with the structure of the food. Therefore, the understanding of the sensory perceptions of food is of interest to the food industry, being important in the design and evaluation of products (van Aken et al., 2005).

While eating, structures of the food are broken down into smaller fragments, thereby releasing entrapped taste and aroma compounds as well as fluids and solid particles. The process of eating is a mechanical, chemical, and enzymatic process. When eating, the visual impressions of the food, the aroma, and the tactile sensations and sounds from touching the food with your

hands, or cutlery, together give a first piece of information of the structures in the food and contributes to the expectations (Kilcast and Lewis, 1990, van Aken et al., 2005, Spence, 2016). Structural arrangements on the surfaces and the rheological properties are important for how the food is visually appreciated (van Aken et al., 2005). Taste and aroma are linked to the solubility of the compounds in different phases, such as water, and oil (Vilgis, 2013). Taste compounds are usually water soluble, while aroma compounds are to a greater extent fat soluble. The temperature of the food affects the solubility and volatility of taste and aroma compounds.

Texture is one of the aspects of food that is most directly linked to the structures in the food. It can be defined using four statements according to Szczesniak: "i) texture is a sensory property, ii) it is a multiparameter attribute, iv) it is detected by several senses, and iv) it derives from the structure of the food" (Szczesniak, 2002). Several different mechanically sensitive tactile receptors in the tongue, teeth, lips and chin, detect the mechanical properties derived from the structures of the food (Foegeding et al., 2011).

The perceived texture of different food materials and whole food products, and especially the fracture pattern of the structures involved, is determined by the interactions between the structural elements. The structure also affects the energy needed for deformation through mastication, i.e. the chewing and swallowing process (Witt and Stokes, 2015). When eating composite foods, the different structures are deformed and fractured differently, and the perception of the texture is complex (Scholten, 2017). The size, shape and hardness of particles in e.g. emulsions and dispersions, have an impact on the sensory perception. Rounder, and softer particles can be of a larger size than hard and sharp-edged ones, before they are detected by the consumer (Scholten, 2017). Sharp-edged particles as small as 10 µm can be detected by human fingertips, and in theory, emulsion droplets of 0.5 µm may be detected in the mouth (Booth, 2005). Particles that are too small to be detected are responsible for the smooth feeling of many food products, for example chocolate. In whey protein gels, even a very small difference in the particle size can be detected by the consumer (Langton et al., 1997). Incorporation of air into food also changes the mouth-feel of the product (Campbell and Mougeot, 1999).

Mouth-feel properties are another aspect of the food that have impact on how it is perceived (Scholten, 2017). Lubrication of the food, the interactions between the surfaces of the food and the oral surfaces, and the interactions with saliva are aspects which affect the mouth-feel. The saliva plays an important role in the mastication; it serves as a lubricant which reduces the friction of the food towards the surfaces of the oral cavity (Foegeding et al., 2011), it is the medium in which the food particles are dispersed, it softens particles, and helps in the aggregation of particles while starches are digested by the amylases present (Witt and Stokes, 2015).

The microstructure of soft and liquid food is important also for the flavor and texture perception, even though no chewing is needed before swallowing. During the relatively short period of time in the mouth, the structure is changed, due to interactions with saliva (Singh et al., 2015). For example, enzymes in the saliva induce aggregation of emulsion droplets in milk, resulting in the formation of slimy strings (van Aken et al., 2005).

Texture can influence nutrition by influencing the acceptance of the product by the costumer, who will most likely choose the most appealing product. Texture also have impact on the particle size of the food reaching the stomach, and on the amount of saliva produced during chewing, which both affects the rate of nutrient absorption and digestion (Kilcast and Lewis, 1990). The bioavailability and the absorption rates of nutrients, as well as the degradation are all affected by the structure of the food material and the matrices therein (Singh et al., 2015). By designing the structure, it is possible to make a healthier food product with reduced levels of fat, salt and sugar, without losing desirable traits of the product, like mouth-feel and flavor release.

Proteins

Proteins are composed of amino acids linked together into polypeptides by peptide bonds (Figure 7). There are 20 proteinogenic amino acids in nature, and they all have different properties, depending on their side chain. In Table 2 the amino acids are listed with the full name, 3-letter code, 1-letter code, chemical formula of the side chain and some of the chemical features. The physical, chemical and biological properties of proteins are determined by the amino acids and their internal order. The amino acid sequence of a polypeptide is called the primary structure (Figure 8, A). Native proteins are folded into a three dimensional structure, which is crucial for the protein's biological function. To reach this structure, the polypeptide will first adapt certain secondary structures depending on the order of the amino acids. Periodical amino acid sequences will give α-helices and β-pleated sheets (Figure 8, B), while more irregular sequences form loops and other disordered structures. The folding of a polypeptide is finished when the tertiary structure is reached. The tertiary structure is the overall assembly of the peptide chain and is mainly stabilized by internal disulfide bonds, hydrogen bonds, hydrophobic and ionic interactions (Figure 8, C). Usually the bioactive protein has a quaternary structure, where several polypeptides, called subunits, form a complex (Figure 8, D) stabilized by hydrogen bonds, hydrophobic and electrostatic interactions (Berg et al., 2005).

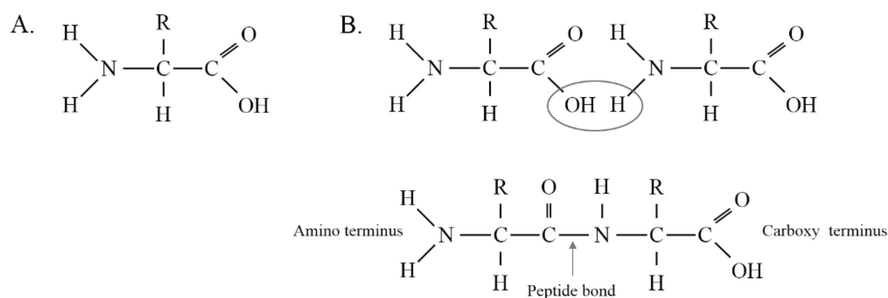

Figure 7. A: The general molecular structure of an amino acid, where R is the side chain. B: The peptide bond linking amino acids into peptides is formed by a condensation reaction.

*Table 2. The 20 natural proteinogenic amino acids, and the chemical formulas of the side chains are presented, as well as some chemical properties: The class of the side chain, the charge at pH 7.4, the isoelectric point (pI), and the normalized hydrophobicity of the amino acids. The abundance of the amino acids in proteins from all kingdoms is given as % of all amino acids in the proteome-pI database (Kozlowski, 2017). The essential amino acids for humans are marked with *. Based on NCBI Amino Acid Explorer (NCBI) and †(Kozlowski, 2017).*

Amino acid			**Side chain**	**Side chain class**	**Charge at pH 7.4**	**pI**	**Hydro-phobicity**	**Abun-dance†**
alanine	Ala	A	$-CH_3$	aliphatic		6.01	0.806	8.76
arginine*	Arg	R	$-(CH_2)_3NH-C(NH)NH_2$	basic	positive	10.76	0.000	5.78
asparagine	Asn	N	$-CH_2CONH_2$	amide	polar	5.41	0.448	3.93
aspartic acid	Asp	D	$-CH_2COOH$	acid	negative	2.85	0.417	5.49
cysteine	Cys	C	$-CH_2SH$	S containing	polar	5.05	0.721	1.38
glutamic acid	Glu	E	$-CH_2CH_2COOH$	acid	negative	3.15	0.458	6.32
glutamine	Gln	Q	$-CH_2CH_2CONH_2$	amide	polar	5.65	0.430	3.9
glycine	Gly	G	-H	aliphatic		6.06	0.770	7.03
histidine*	His	H	$-CH_2-C_3H_3N_2$	basic aromatic	positive	7.60	0.548	2.26
isoleucine*	Ile	I	$-CH(CH_3)CH_2CH_3$	aliphatic		6.05	1.000	5.49
leucine*	Leu	L	$-CH_2CH(CH_3)_2$	aliphatic		6.01	0.918	9.68
lysine*	Lys	K	$-(CH_2)_4NH_2$	aliphatic	positive	9.60	0.263	5.19
methionine*	Met	M	$-CH_2CH_2SCH_3$	S containing		5.74	0.811	2.32
phenylalanine*	Phe	F	$-CH_2C_6H_5$	aromatic		5.49	0.951	3.87
proline	Pro	P	$-CH_2CH_2CH_2-$	cyclic		6.30	0.678	5.02
serine	Ser	S	$-CH_2OH$	-OH	polar	5.68	0.601	7.14
threonine*	Thr	T	$-CH(OH)CH_3$	-OH	polar	5.60	0.634	5.53
tryptophan*	Trp	W	$-CH_2C_8H_6N$	aromatic		5.89	0.854	1.25
tyrosine	Tyr	Y	$-CH_2-C_6H_4OH$	aromatic	polar	5.64	0.714	2.91
valine*	Val	V	$-CH(CH_3)_2$	aliphatic		6.00	0.923	6.73

The folded structure of the protein can be stabilized with disulfide bridges between cysteines, hydrogen bonds, electrostatic and hydrophobic interactions. By disrupting the intra-protein amino acid interactions the protein will unfold and become denatured. In several applications, for example in many protein purification methods, it is necessary to interrupt the folded structure. Urea and sodium dodecyl sulfate (SDS) are compounds commonly used for disrupting all non-covalent bonds in the protein and between proteins, while reducing agents like β-mercaptoethanol break disulfide bridges (Berg et al., 2005). Unfolding might cause the proteins to precipitate due to lower solubility, making it possible to separate them. The protein structure can also be disrupted by heat, detergents, high salt concentrations and an environment with a pH close to the isoelectric point of the protein. The isoelectric point is where all the net charge of the protein is zero.

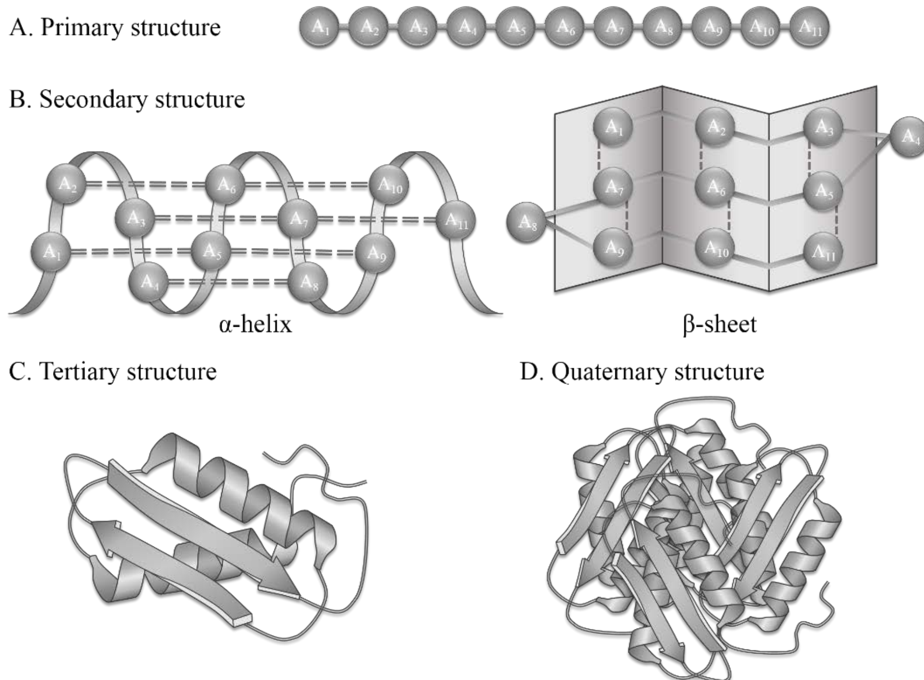

Figure 8. The levels of protein structure. A: Primary structure: Amino acids in sequence. B: Secondary structure: The chain of amino acids folds into α-helices and β-sheets. C: Tertiary structure: The partly folded amino acid chain adapts a three dimensional structure. D: Quaternary structure: Several chains, called subunits, form a complex.

RuBisCO – the world's most abundant protein

Ribulose-1,5-bisphosphate carboxylase/oxygenase, or RuBisCO, is an enzyme present in all higher plants and also in cyanobacteria, phytoplankton and algae (Andersson and Backlund, 2008). The protein has a key role in photosynthesis, where it catalyzes the primary binding of CO_2. In green leaves RuBisCO is the most abundant protein, comprising up to 50 % of the soluble proteins in the leaf, which makes RuBisCO one of the most plentiful proteins in the biosphere. The protein is located in the stroma of the chloroplasts in the plant cell (Ellis, 1979). The proteins in green leaves are divided into green and white fraction proteins, with the white fraction mainly composed of RuBisCO. When RuBisCO first was discovered it was termed Fraction I protein (Wildman and Bonner, 1947).

The RuBisCO protein in plants is hexadecameric in its form, consisting of 8 large (L) and 8 small (S) subunits, with molecular weights of 55 kDa and 12.5 kDa, respectively. Four L2 dimers together form a barrel-like spherical structure with the small subunits on the top and bottom, the structure of the native protein is shown in Figure 9 (Andersson and Backlund, 2008, Douillard and De Mathan, 1994). From X-ray scattering, the outer and inner radius of the structure have been established to 56.4 Å and 14.3 Å, respectively (Donnelly et al., 1984). The dimers interact through eight salt links per dimer-dimer interface. The salt links between the subunits are disrupted with SDS, and they are separated in SDS-PAGE (Onaizi et al., 2007). In Table 3 the amino acid compositions for the two subunits of spinach RuBisCO are compiled. The native protein need Mg ions as cofactors for its enzymatic activity (Ellis, 1979). The tertiary structure of RuBisCO from different species are highly conserved, especially the large subunit, which makes up the catalytic site (Andersson and Backlund, 2008).

The calculated theoretical isoelectric point of spinach RuBisCO is 6.13 for the large subunit, 6.03 for the small subunit and 6.03 for the total protein (Uniprot entries P00870 and P00870 (www.uniprot.org), the calculations were done using Expasy, ProtParam (web.expasy.org/cgi-bin/protparam/ protparam)). A study on lucerne RuBisCO reported that a pure protein solution got turbid below pH 6.5 and that the proteins precipitated at pH 5. The proteins redissolved at pH below 5 and the solution was clear again at pH 3.4 (Tomimatsu, 1980). The pH values used for acid precipitation of the protein in other studies are ranging between 3.5 and 4.5 (Figure 11).

The pH and the salt concentration of the solute have impact on the denaturation temperature of RuBisCO. At a higher ionic strength the denaturing temperature was increased from 76.2 ˚C to 79 ˚C for lucerne RuBisCO, while at pH 4, no thermal denaturation occurred, probably due to an unfolded structure at that pH (Tomimatsu, 1980). Other reported denaturation temperatures for RuBisCO from lucerne are 67.15 ˚C at pH 7.5

and 66.45 ˚C at pH 10.1 (Béghin et al., 1993). RuBisCO from spinach has been reported to denature at 64.9 ˚C (Martin et al., 2014).

The nitrogen content in RuBisCO from lucerne has been reported to be 17.0 % (Tomimatsu, 1980). The theoretical nitrogen content of RuBisCO from spinach is 16.9 % (Uniprot entries P00870 and P00870 (www.uniprot.org), the calculations were done with Expasy, ProtParam (web.expasy.org/cgi-bin/protparam/protparam)).

Table 3. Amino acid composition of the large and small subunit of RuBisCO from spinach, both as the absolute number of each amino acid and the fraction compared to the total subunit. Uniprot entries P00870 and P00870 (www.uniprot.org/), the calculations were done using Expasy, ProtParam (web.expasy.org/cgi-bin/protparam/protparam).

Amino acid	Large subunit		Small subunit	
Ala	43	9.10%	6	4.90%
Arg	29	6.10%	4	3.30%
Asn	16	3.40%	3	2.40%
Asp	26	5.50%	6	4.90%
Cys	9	1.90%	1	0.80%
Gln	11	2.30%	4	3.30%
Glu	33	6.90%	10	8.10%
Gly	45	9.50%	9	7.30%
His	15	3.20%	1	0.80%
Ile	19	4.00%	4	3.30%
Leu	41	8.60%	11	8.90%
Lys	23	4.80%	10	8.10%
Met	10	2.10%	3	2.40%
Phe	20	4.20%	8	6.50%
Pro	22	4.60%	12	9.80%
Ser	18	3.80%	3	2.40%
Thr	34	7.20%	5	4.10%
Trp	8	1.70%	4	3.30%
Tyr	19	4.00%	8	6.50%
Val	34	7.20%	11	8.90%
Total	475		123	

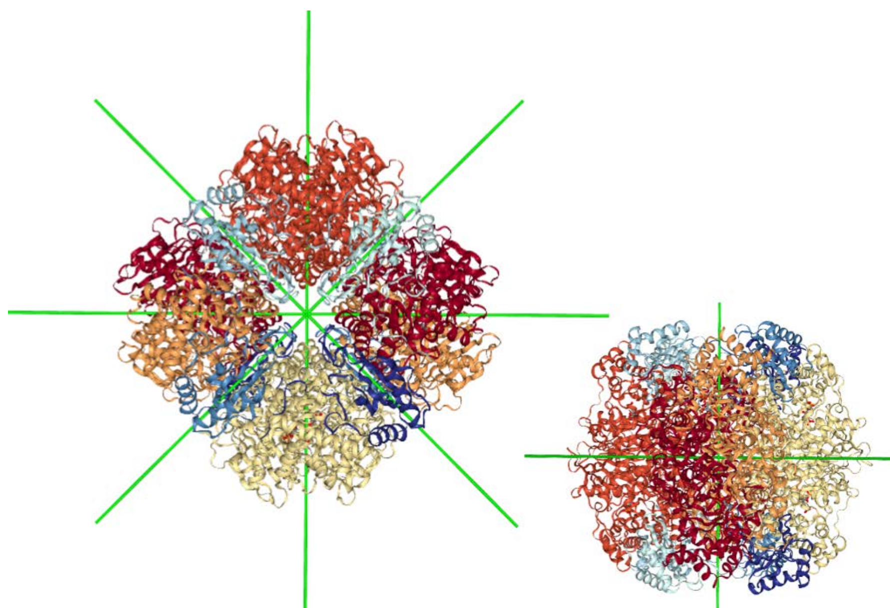

Figure 9. RuBisCO from spinach. Image from the RCSB PDB (www.rscb.org) of PDB ID 8RUC (Andersson, 1996).

Leaf proteins, and more specifically RuBisCO, was proposed as a potential source of food protein for human consumption already in the 1940's (Pirie, 1942). It is widely available in green waste material in agriculture and in nature. The amino acid composition of the protein is favorable for human consumption, since almost all the essential amino acids are present in high enough amounts, as is shown in Table 4 (de Jong and Nieuwland, 2011). Animal studies have confirmed the digestibility of leaf protein concentrates (Carlsson and Hanczakowski, 1985, Subba Rau et al., 1972), and a study on the nutritional value of leaf protein concentrates as food supplements for children have shown effects comparable with those of milk (Shah et al., 1980). Even more interesting in food applications are the techno-functional properties of the protein. Native RuBisCO has been shown to be a good gelling and foaming agent, and fibrillary structures with meat-like texture have been made (Pouvreau et al., 2014).

Table 4. Essential amino acid composition of RuBisCO (all subunits) from different species given as percentage of the required amount for preschool-age children. Adapted from (de Jong and Nieuwland, 2011)

Essential amino acid	Spinach (*spinacia oleracea*)	Tobacco (*nicotiana tabacum*)	Lucerne (*medicago sativa*)
Ile	86.9	102	106.2
Leu	130.2	130	128
Lys	98.7	111.7	105.6
Met + Cys	181.4	151.7	161.8
Phe + Tyr	202.2	198.2	201.4
Thr	181.5	150.4	159.9
Trp	302.4	327.3	303.3
Val	172.9	189.6	169.2

Plant protein extraction methods

Proteins in green leaves were first isolated by the French biochemist H.M. Rouelle in 1773, even though proteins were not fully understood at that time (Pirie, 1987). Using a mortar and pestle he pressed out juice from leaves (green juice, or GJ) and created a green coagulum by first heating the solution until it no longer was possible to keep a finger in the solution for any extended time. He filtered away the green coagulum, and heated the filtrate, the brown juice (BJ) further, which gave a light coagulum. The methods have been developed considerably since then, but similar underlying principles are still adopted; extract the green juice from the leaves, remove the green fraction, and finally purify and concentrate the white fraction. The basic principles of leaf protein extraction are illustrated in Figure 10 and a more detailed overview of some of the methods used in the literature is presented in Figure 11.

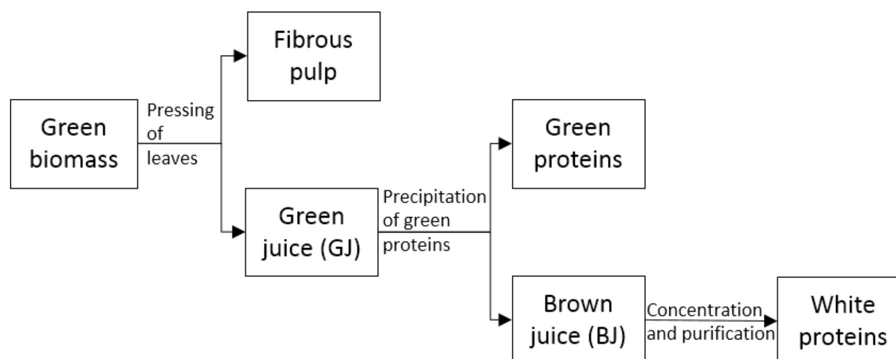

Figure 10. The basic principles of protein extraction from green biomass.

Pressing of green juice

RuBisCO is a water soluble protein located inside the leaf cells, and the first step in the protein extraction process is to disrupt the cell walls to release intercellular fluids. To reach a high protein yield it is crucial to get a high level of cell fragmentation (Betschart and Kinsella, 1973). One efficient method for obtaining green juice from fresh leaves is screw pressing, both lab scale and pilot scale (Colas et al., 2013b, Edwards et al., 1975, Bray and Humphries, 1979). In a screw press, fluids are pressed out from the leaves and the fibrous pulp is extruded at the end of the screw. By choosing a suitable screw press it is possible to get out 70 % or more of the green juice in the leaves, and by adding water during the process, proteins trapped in the fibrous pulp are washed out, resulting in higher protein recovery (Colas et al., 2013a). Ultrasonication of homogenized cauliflower leaf material have recently been shown to result in a more extensive cell disruption, thereby leading to an increased protein recovery (Xu et al., 2017). Increased yields can also be achieved by using high pH solutions during the juice extraction, due to an increase in protein solubility and more efficient disruption of chloroplasts in alkaline environments (Betschart and Kinsella, 1973). Fresh, or frozen, leaves

seem to be the most common raw material, but there are studies where dried leaf material is used. In such cases alkali solubilization of the protein at high pH, e.g. 10, increases the yields significantly (Hojilla-Evangelista et al., 2017).

The protein recovery can also be increased by the addition of sodium metabisulfite ($Na_2S_2O_5$) in the juicing process, a preservative and antioxidant compound (E-number E223, (Livsmedelsverket, 2017)), which also reduces browning reactions (Edwards et al., 1975, Fiorentini and Galoppini, 1981, Martin et al., 2014). Detergents, such as Tween 80, are amphipathic and interrupt ordered structures in the cell membranes leading to the release of membrane bound proteins (Dotsenko and Lange, 2017). This leads to higher protein yields of protein extractions from leaves, however it is not stated weather these proteins have positive effects on a food protein concentrate. The use of proteases in the extraction resulted in higher total protein yields, but it is worth mentioning that only degraded peptides of varying sizes remained (Dotsenko and Lange, 2017). Addition of different buffer solutions containing reducing agents and sucrose when juicing leaves have also been shown positive for the protein extractability, but it also adds extra cost to the process and may affect the quality of the end product.

Separation of green proteins

The green juice contains not only soluble proteins, but also chlorophyll, chlorophyll related proteins, membrane fragments and other unwanted compounds, which influences the quality of the protein concentrate. By removing the green fraction, the functional properties of the white fraction are improved, and the green color and the grassy smells and tastes are reduced. Proteins in green leaves precipitate at different temperatures, which can be utilized in the fractionation process. Proteins in the green fraction aggregate at temperatures ranging between 50 and 65 °C, and the soluble white proteins at temperatures of 80-82 °C (Tamayo Tenorio et al., 2016). Sequential heating with an intermediate separation step has been proposed as a way of fractioning the proteins. Thermal treatment of GJ at 60 °C for 20 seconds by steam injection was shown to be enough to cause coagulation of the green fraction (Edwards et al., 1975), while milder treatments at lower temperatures require more time, e.g. 50 °C for 30 minutes (Martin et al., 2014). Figure 11 shows some other temperatures, as well as complete processes, used in the literature for protein extraction from lucerne, sugar beet leaves, spinach, rye grass, tall fescue, water plants and some photosynthetically active unicellular organisms. Another method for removing the green fraction is the use of flocculants which cause larger particles to sediment (Bray and Humphries, 1979, Fiorentini and Galoppini, 1981). The aggregated proteins are easily removed by centrifugation or filtration leaving a clear brown supernatant.

Concentration and purification of white proteins

After the removal of the green fraction, a brown juice containing the white proteins is obtained. Since many of the compounds present in the brown juice might affect the properties of the proteins, both functional and nutritional, a purification might be needed, depending on the intended use of the protein concentrate. Salt fractionation in combination with chromatographic methods (Sarkar et al., 1975, Martin et al., 2014) and membrane filtration techniques (Zhang et al., 2015, Firdaous et al., 2017), can give relatively pure concentrates. Both chromatography and membrane filtration has long been regarded too expensive for large scale processing, but the techniques are becoming cheaper and more efficient. Another less advanced method is isoelectric precipitation. At pH values close to the isoelectric point of the proteins they precipitate and can be separated from the soluble compounds. For the white protein fraction, pH values of 3.5 to 4.5 have been used (Bray and Humphries, 1979, Merodio and Sabater, 1988, Lamsal et al., 2007, Kobbi et al., 2016). The precipitate can be washed with pH-adjusted water to remove some of the co-precipitated compounds, and thereafter redissolved in neutral or slightly alkaline water (Lamsal et al., 2007). If a purer concentrate without salts and other small molecules is wanted, a dialysis step can be added in the process (Hojilla-Evangelista et al., 2017). Thermal denaturation by heating the white fraction to 95-100 ˚C is an option for concentration, and the simultaneous pasteurization serves to prolong the storage time (Merodio et al., 1983).

The protein yields when recovering only the white fraction are normally low, especially if high purity is desired. When extracting RuBisCO with 90 % purity, 1 g protein was gained from 10 kg of fresh spinach (Martin et al., 2014). In order to get higher yields, and also a less complex process, total leaf protein concentrates containing both the green and white fraction proteins, is one option (Tamayo Tenorio et al., 2016). Total protein concentrates are produced by full precipitation of the GJ, which can be done by heating at 80 ˚C (Dewanji et al., 1997), or acidification, either by addition of hydrochloric acid (Merodio and Sabater, 1988) or by fermentation (Santamaría-Fernández et al., 2017, Hermansen et al., 2017). When fermenting GJ, naturally occurring microorganisms, or inoculated lactic bacteria, reduce the pH to around 3.5, resulting in the precipitation of most proteins. Precipitation of unfractioned proteins is also an option carried out by freezing and subsequent thawing of GJ, resulting in a freezing curd composed mainly of chloroplasts (Hernández et al., 1998). Ultrafiltration of GJ is another possibility to get a concentrated protein solution (Koschuh et al., 2004).

Large scale production

The first serious interest for large scale production of leaf protein concentrates (LPC) was raised in the 1940's in Britain as a proposed way to ensure the availability of food protein during world war II (Pirie, 1966). During the last decades several different pilot plants have been set up, both as pure protein factories (Edwards et al., 1975, Fiorentini and Galoppini, 1981) and extraction processes in combination with biogas plants (Hermansen et al., 2017, Santamaría-Fernández et al., 2017) or ethanol plants (Chiesa and Gnansounou, 2011). Low yields in the extraction process have been an issue when trying to make industrially scaled processes, and biorefineries where valuable chemical compounds, except from biogas and ethanol, are extracted from green biomass is a proposed way of making the process profitable (Kamm et al., 2016).

Other concerns in protein extraction

Polyphenol oxidases and phenolic compounds in the plant cell are mixed when the cell is disrupted in the juice extraction process, leading to the formation of reactive quinones (Kroll et al., 2003). Quinones can bind covalently to proteins and impair the nutritive value of the proteins and change their functional properties. Enzymatic browning of food products are mainly due to these reactions. By using buffers containing reducing agents and antioxidants, such as metabisulfite ($Na_2S_2O_5$) (Fiorentini and Galoppini, 1981, Edwards et al., 1975, Martin et al., 2014), the occurrence of these reactions may be reduced, resulting in increased protein yields and stability of the resulting product. Phenolic compounds can be removed by adsorptive resins (Firdaous et al., 2017), or by chromatographic separation (Sarkar et al., 1975). Low temperatures during the protein extraction process and minimized contact with oxygen also reduces the browning reactions.

Cell disruption also results in the release of enzymes with proteolytic activity, which can have adverse effects on the stability of proteins in the GJ as well as of the protein concentrates. RuBisCO in fresh lucerne GJ was degraded completely after 72 hours at 20 °C, and 30 % of the RuBisCO had been degraded already after 3 hours (Koschuh et al., 2004). When GJ was stored at 4 °C as much as 80 % of the RuBisCO was still intact after 24 hours. The activity of the enzymes might, however, be lost in the heating steps in the protein extraction, e.g. activity of an aminopeptidase and a proline iminopeptidase in cabbage leaf protein extracts decreased after heat treatment at 50 °C (Marinova et al., 2008).

Leaf material

Lucerne (*Medico sativa*), also known as alfalfa, has been used as a raw material for protein extraction in several publications (Hojilla-Evangelista et al., 2017, Fiorentini and Galoppini, 1981, Edwards et al., 1975, Lamsal et al., 2007, Kobbi et al., 2016). Other raw materials, not exclusively, are sugar beet

leaves (Merodio and Sabater, 1988, Tamayo Tenorio et al., 2016), tropical aquatic plants (Dewanji et al., 1997), spinach (Martin et al., 2014), rye grass (Bray and Humphries, 1979, Koschuh et al., 2004), photosynthetically active unicellular organisms (Teuling et al., 2017), cauliflower by-products (Xu et al., 2017), and potato haulm (Carruthers and Pirie, 1975).

For the extraction of protein from green leaves, the choice of species are of importance. Availability of material, presence of antinutritional factors, amount of extractable proteins, and toughness of the plant cells are just a few aspects that should be taken into consideration (Pirie, 1987). The species and age of the plant, as well as the time of day, moisture level and fertilization, affects the yield. The optimum stage for harvesting of lucerne is pre-flowering (Fiorentini and Galoppini, 1981). In sugar beet leaves the age of the plant was shown to affect the quantity and quality of the extracted protein, but no clear differences were found for the total protein content (Kiskini et al., 2016). The total phenolic content has been reported to be doubled in leaves from eight months old sugar beets compared to three months old plants (Vissers et al., 2017).

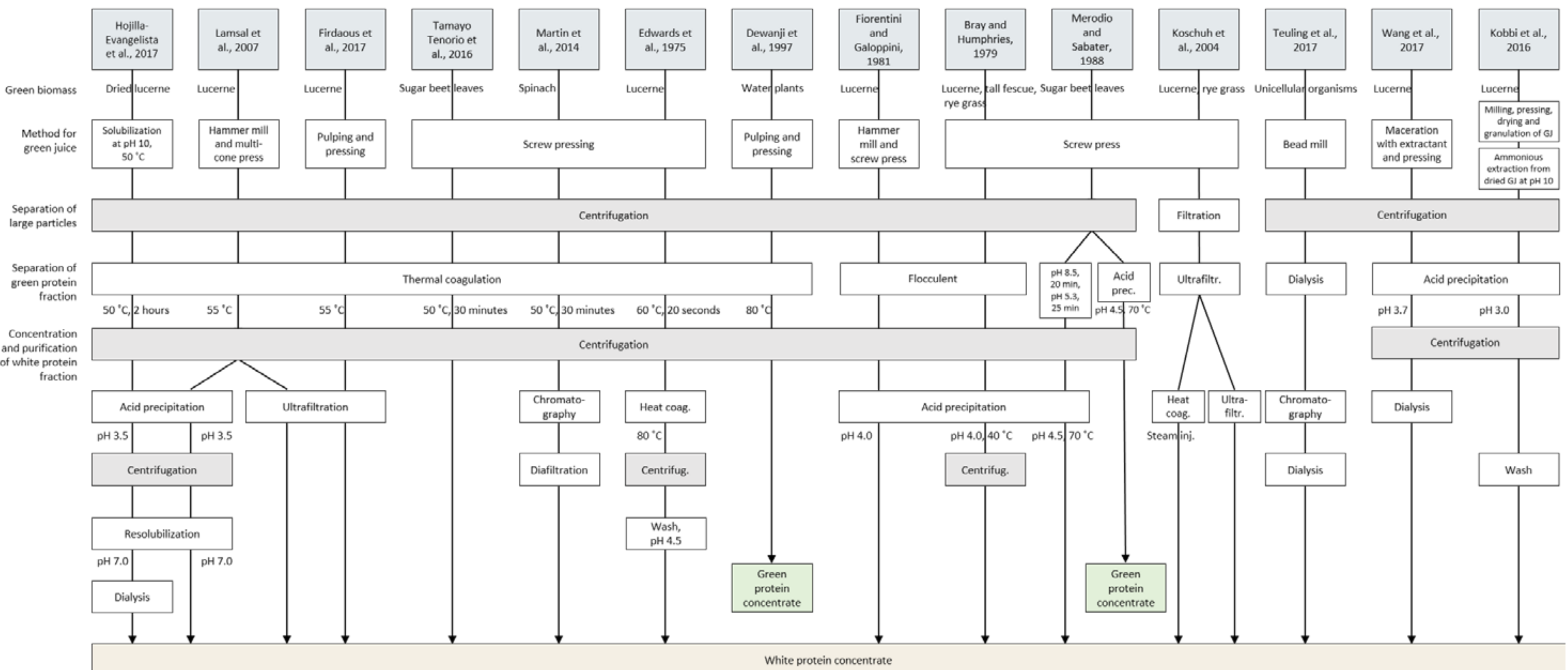

Figure 11. Some protein extraction processes used in the literature.

Methods for characterizing food structures and proteins therein

Foaming properties

The ability of a protein solution or dispersion to form a stable wet foam is regarded as an important functional property. Foam studies have been performed for several kinds of proteins, and some consensus regarding the methods used have been established. Studies have been made on the foaming properties of proteins from, for example, seaweed proteins (Garcia-Vaquero et al., 2016), ultra sound-treated soy protein isolates (Morales et al., 2015), marama bean protein (Gulzar et al., 2017), corn protein (Myers et al., 1994), potato protein (van Koningsveld et al., 2002), alfalfa (or lucerne) (Lamsal et al., 2007), chia seed (Timilsena et al., 2016), lupin (Burgos-Díaz et al., 2016), whey protein and egg white (Pernell et al., 2002). In all of these studies, foams were prepared by introducing air through whipping or mixing. The foam-ability of a protein can be presented in terms of overrun, which is the volume of gas per unit of volume of gas-free material (Campbell and Mougeot, 1999). Foam overrun, or foam capacity, can be calculated using the following formula:

$$FO\ (\%) = \frac{m_2/V - m_1/V}{m_{1/V}} \times 100\ ,$$

where m_1 is the mass of a specified volume V of the solution, and m_2 is the mass of a volume V of the foam. Another commonly used method to assess the foam capacity is to measure the volume of the foam, which might be advantageous for smaller sample volumes (Myers et al., 1994). For solid foams, such as freeze-dried foams, the term volumetric air content is used instead of overrun to describe the relative amounts of gas and solid material (Campbell and Mougeot, 1999).

Foam stability is another interesting parameter when studying wet foams, it is usually expressed as the percentage of foam volume decrease after a specific amount of time:

$$FS\ (\%) = \frac{V_3 - V_1}{V_2 - V_1} \times 100,$$

where V_1 is the start volume, V_2 is the foam volume at the end of aeration, and V_3 is the volume after a specific time (Burgos-Díaz et al., 2016).

Interfacial interactions

To get a deeper understanding of the underlying explanations for different foaming properties among proteins, several approaches have been taken. The protein interactions at, and with, the air-water interfaces in a wet foam are crucial in the foam formation and stability, and elaborate methods have been developed in order to investigate these interactions. Proteins adsorbing to and

desorbing from an interface readily affect its physical properties, and by monitoring for example surface tension and rheological factors, it is possible to get insight into the process.

The surface and interfacial tension can be measured using various instruments, for example a drop profile analysis tensiometer (Mitropoulos et al., 2014), or a contact angle goniometer (Pernell et al., 2002). The rheological properties of the liquid films separating air bubbles in foams are determined by the properties of the liquid phase, but also by the behavior of the proteins at the interfaces. When proteins, for example β-lactoglobulin, as in a study by Lee et al., adsorb to the air-water interface, the microrheological properties are changed, and the adsorption process can be monitored, either through active or passive rheological tests (Lee et al., 2010). Shear oscillation measurements can reveal additional rheological properties of the protein layer at the interfaces, and might be a useful approach (Mitropoulos et al., 2014). Generally, by investigating the rheological properties of wet foams, gels, or other food solutions and dispersions, it is possible to get some insight into the microstructures, and the interactions between the structural elements.

At the adsorption to an air-water interface, the conformation of the proteins may be altered. The structure might also change as a result of different treatments. Changes in the secondary structure of the proteins at interfaces, but also in solid materials, can be analyzed using spectroscopic techniques such as circular dichroism (CD) spectroscopy, Fourier transform infrared analysis (FTIR) and nuclear magnetic resonance (NMR) (Hammann and Schmid, 2014), but also through rheological studies (Noskov, 2014). CD spectroscopic methods utilize the optical activity of most amino acids, and changes in the secondary structure will lead to changes in the CD spectrum, e.g., changes in the ellipticity (Hammann and Schmid, 2014). External reflection circular dichroism, ERCD, has been used for studying conformational changes at the air-water interface for β-lactoglobulin (de Jongh and Meinders, 2002). In ERCD the reflection of the interfaces is considered and compensated for. FTIR has successfully been used to study the secondary structure of wheat gluten proteins in freeze-dried foams (Wu et al., 2017, Blomfeldt et al., 2012). A fluorescence based method was recently proposed, where the fluorescence of a dye, Nile red, in hydrophobic environments was utilized to monitor protein unfolding at an air-liquid interface (Leiske et al., 2016). Another method suitable for investigating interfacial structures is atomic force microscopy, AFM, with which it is possible to image the protein networks at the interface through topographic mapping (Gunning and Morris, 2017).

Imaging of food structures

During the last decades sophisticated X-ray techniques have emerged, providing powerful ways to investigate food structures, both at a molecular and macroscopic level. The adsorption process of proteins at air-water interfaces have for example been investigated using a simultaneous multiple-angle-wavelength-dispersive X-ray reflectometer at a synchrotron beam line (Yano et al., 2008, Yano et al., 2013). By this a density profile along the surface normally can be obtained. Small-angle and wide-angle X-ray scattering are other examples of X-ray techniques used in characterizing materials and macromolecules, they can both provide information on morphology and crystallinity of materials (Hammann and Schmid, 2014). Small-angle X-ray scattering has been used to characterize freeze-dried foams (Wu et al., 2016, Blomfeldt et al., 2012).

Through X-ray techniques such as ptychographic X-ray computed tomography, PXCT, and X-ray micro-computed tomography, µCT, it is possible to get 3D images of materials, such as food products. PXCT, has been successfully applied in imaging emulsions, and it is a promising technique for imaging complex food structures (Nielsen et al., 2016). µCT has been used for imaging aerated food products, for example bubbles encapsulated in chocolate (Haedelt et al., 2007), and aerated chocolate bars (Lim and Barigou, 2004). The methods can both provide a detailed 3D image of the structure without any staining needed, and the composition of the sample can be assessed. Both synchrotron µCT systems and laboratory setups are available, which gives it some advantage over other X-ray imaging techniques (Schoeman et al., 2016).

Besides light microscopy, several electron microscopy methods have been used in high-resolution imaging of food structures. Scanning electron microscopy, SEM, has been used to observe the structure of freeze-dried food products (Ciurzyńska et al., 2017, Carvalho et al., 2017). SEM has also been used to study freeze-dried gluten foams (Wu et al., 2017). Field-emission scanning electron microscopy, FE-SEM, can give even higher resolution in some cases, and have been used to study freeze-dried foams from gluten (Blomfeldt et al., 2012). Cryo-SEM, environmental SEM, and transmission electron microscopy, TEM, are other examples of methods widely used (Aguilera and Stanley, 1999).

Confocal laser scanning microscopy, CLSM, is another possibility to get 3D images of food structures (Lorén et al., 2007). In confocal microscopy the fluorescence of staining molecules binding to different compounds in the structure is captured, and by moving the focal plane it is possible to obtain a 3D image. Immunostaining with fluorochrome-labeled antibodies specific for the protein of interest provides even more information. In one study on freeze-dried gluten foams, the different protein fractions in thin sections of the foam could be targeted, and the distribution of the fractions in the foams could be

imaged (Blomfeldt et al., 2012). Wet egg white and whey foams have been studied using CLSM (Pernell et al., 2002), and 3D images of emulsions have been made through staining of lipids (Nielsen et al., 2016).

In order to get data from images, proper image analysis is crucial. Through different image analyses it is possible to get information on parameters such as bubble size, particle size, size distribution, wall thickness, protein distribution and much more (Aguilera and Stanley, 1999). More or less automated image analysis approaches have been developed, for example the FoamScan® system from Teclis Instruments, France, which analyses the drainage rate, stability and bubble size distribution (TeclisScientific, 2017, Wang et al., 2017).

Protein determination

Electrophoretic techniques, such as SDS-PAGE, can be a useful and relatively easy way to get insight into the distribution of proteins in the protein isolates (Burgos-Díaz et al., 2016, Garcia-Vaquero et al., 2016, Kobbi et al., 2016). By separating proteins according to their molecular size, a qualitative analysis is possible. Two-dimensional electrophoresis (2DE) separates proteins both according to their isoelectric point and their molecular size, and may contribute to a more reliable identification (Burgos-Díaz et al., 2016). Immunoblotting, or other immunotechnological methods, with antibodies specific for the target protein offers another even better possibility to identify the protein, and has successfully been used to detect RuBisCO proteins (Teuling et al., 2017). SDS-PAGE can also be used to investigate if polymerization has occurred (Hammann and Schmid, 2014).

Size-exclusion high performance liquid chromatography, SE-HPLC, can give information on the protein amount and size distribution, similarly to SDS-PAGE (Johansson et al., 2001). By using a sequential protein extraction procedure on the material, the different fractions of proteins can be assessed, and the interactions between the proteins can also to some extent be investigated, e.g. if polymerization has occurred the kind of bonds can be assessed. Reversed phase high performance liquid chromatography, RP-HPLC, is another useful chromatographic method for assessing polymerization (Kuktaite et al., 2004). In RP-HPLC the molecules are separated due to their hydrophobicity rather than size.

Sensory testing

Even though instrumental tests offer a controlled environment and a high reproducibility in examining food, human sensory panels are the best instruments for measuring human perceptions of food. A sensory panel requires long training, but after correct calibration it is hard to replace with machines (Foegeding et al., 2011). Sensory testing in combination with imaging methods and/or mechanical testing can give deep understanding of how the food structure and texture perception are linked, and experimental

setups where that is used can be found throughout the literature. One example is a study on how bubbles in aerated chocolate affect the experience of the chocolate (Haedelt et al., 2007).

Attempts have been made to overcome the subjective aspects of using a human sensory panel by combining test persons with instruments. One such method to assess the mechanical properties is electromyographic analysis, where the jaw muscle movements are recorded, giving information on the chewing patterns when eating a specific product (Foegeding et al., 2006).

Instrumental testing of food perception

The mechanical properties of the food are in many ways linked to the structure of the food, and also the texture. Food can in many aspects be studied with the same, or similar, methods as are used in material science. Compression tests are used to assess the mechanical properties of materials, such as freeze-dried gluten foams (Wu et al., 2016, Blomfeldt et al., 2012) and may also give a first idea of the textural properties of a freeze-dried food product (Ciurzyńska et al., 2017). Mechanical testing, such as a three-point bending test can give information of the flexural stress at break and flexural modulus of freeze-dried gluten foams (Wu et al., 2017), and the same technique has been used to assess the mechanical properties of dense corn extrusions (Chaunier et al., 2007). Rheological tests can be used for studying the microstructures in food as mentioned earlier, but it is also a valuable tool in the assessment of food texture (Foegeding, 2007). Tribology measurements are rheological tests where properties of the mouth, such as the roughness of the tongue, have been mimicked (Chen and Stokes, 2012).

Other aspects of the food that affect how it is perceived, are the visual aspects, taste and fragrance. Color can be measured using a portable spectrophotometer (Ciurzyńska et al., 2017, Carvalho et al., 2017), and chromatographic methods such as liquid and gas chromatography (LC and GC) can be used to track down the compounds responsible for taste and smell. By using GC olfactometry, the compounds responsible for aroma can be detected (Semmelroch and Grosch, 1995). The volatile compounds in the head space of the food are captured, separated in the GC column and detected in parallel by a trained test person and a mass spectrometer.

Freeze-drying as a food production operation

Freeze-drying, or lyophilization, is a process where a solvent, usually water, is removed from a pre-frozen material through sublimation at low temperature and pressure (Ciurzyńska and Lenart, 2011). The process offers a gentle way to dry sensitive materials, such as pharmaceuticals and bioactive proteins, but it is also widely used in the food industry, for example in the production of instant coffee. The resulting freeze-dried product generally has its overall structure and shape preserved, but the ice crystal formation in the first freezing step might alter it. The size of the ice crystals formed depends on the freezing rate, at higher rates larger crystals will form. The shape of the crystals can be controlled through directed solidification, which can been utilized in freeze-casting of porous materials (Deville, 2010). A kind of freeze-casting has been used to produce wheat gluten foams through freeze-drying of gluten dispersions in molds (Wu et al., 2016). Porous gelatin hydrogels (Van Vlierberghe et al., 2007) and hydrocolloid gels (Ciurzyńska et al., 2017) have been made in similar ways.

Freeze-drying of food products alter the textural perception, probably due to a changed microstructure. The texture of freeze-dried yoghurt foams was studied by Carvalho et al., and in comparison to air dried foams, freeze-dried foams were perceived as more soggy (Carvalho et al., 2017). Freeze-drying of yogurt foams resulted in smaller pores with thinner cell walls compared to air-dried foams, and overall a more porous structure. Both the higher porosity and the thinner walls contribute to a quicker dehydration, which possibly explains the change in texture.

Conclusion

Proteins are important compounds in many food products not only due to their nutritional value, but also for their functional properties. The functional properties of the proteins have considerable impact on the internal structures in the food, determining the perceived attributes of the food. In the emerging market of plant based options to animal protein sources, leaf proteins have great possibilities as a food ingredient in a near future. The concept of using green leaves as a protein source has been around for several decades (Pirie, 1942), but during the last years interest has been renewed. A substantial number of studies regarding leaf protein extraction both as a sole product (Kiskini et al., 2016, Kobbi et al., 2016, van de Velde et al., 2011, Tamayo Tenorio et al., 2016), and as part of a biorefinery process (Santamaría-Fernández et al., 2017, Hermansen et al., 2017, Chiesa and Gnansounou, 2011) has been reported lately. The functional properties of the main leaf protein RuBisCO has also been investigated to some extent (Martin et al., 2014, Lamsal et al., 2007, Hojilla-Evangelista et al., 2017).

In this introductory paper some of the reported leaf protein extraction methods have been assembled. Through this study the need for better, simpler and more efficient methods has become apparent. The analytical techniques mentioned may all contribute to understanding how leaf proteins, and especially RuBisCO, perform in food applications.

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