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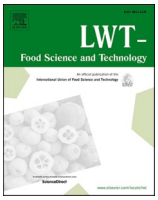

Applicability of leaf protein concentrates from various sources in food: Solubility at food-relevant pH values and air-water interfacial properties

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ABSTRACT

Leaf protein concentrates (LPCs) from green leafy biomass have potential as a sustainable protein source. Here, protein composition, solubility, foamability, and interfacial properties of LPCs from six different biomass types (beetroot, kale, lucerne, mangold, spinach, and sugarbeet) were evaluated. Although the LPCs showed some differences in protein composition and solubility, the foamability and interfacial properties were strikingly similar. RuBisCO was the major protein in all LPCs. All LPCs demonstrated lower solubility at pH 5.0 and 3.0 than at pH 7.0. Both the highest and the lowest solubility was found for sugarbeet: 67.6 ± 7.9% at pH 7.0 and 9.2 ± 4.0% at pH 5.0. The LPCs formed aggregates of increasing size at pH 3–4.5, matching the solubility results. The LPCs stabilized foams in a preliminary foaming test, although the main focus was on evaluating air-water interfacial properties. The ability of the proteins to diffuse to and adsorb at the interface and their ability to form viscoelastic films thereat, using an optical tensiometry method, was investigated. All six LPCs showed an interfacial behavior resembling that of reference egg white protein (0.1–1.0 mg/ml). Our findings indicate good opportunities for using LPCs in food applications and in particular as an alternative to egg white in foams.

1. Introductions

Plant-based protein sources that can deliver sustainable and highly nutritious food are of outmost importance; climate related food challenges in combination with an increasing global population call for changes in our food habit (Mbow et al., 2019). Green agricultural biomass is a promising plant protein source, given its abundance both in the form of main crops in crop rotation systems (Hansson, Svensson, & Prade, 2021) and as leafy side-streams (Berndtsson et al., 2019; Prade et al., 2021). Green biomass can also be considered a locally produced food resource, thereby reducing transportation needs, as well as meeting the increasing consumer demand for local produce (Nemes et al., 2021). The main protein (~50%) in green biomass is ribulose-1, 5-bisphosphate-carboxylase/oxidase (RuBisCO), an enzyme active in photosynthesis, and thereby present in all photosynthetic organisms (Andersson & Backlund, 2008). Leaf protein extracts consisting mainly of water soluble proteins, with RuBisCO as the main component, are often referred to as the white protein fraction (Nynäs, Newson, & Johansson, 2021), although leaf protein concentrate (LPC) is probably a better term to avoid misinterpretations regarding color (Nynäs, 2022). LPCs have been shown to have several properties that contribute to their potential applicability in foods, including a favorable amino acid composition, good water solubility, and high surface activity (Ducrocq et al., 2022; Hojilla-Evangelista, Selling, Hatfield, & Digman, 2016; Knuckles & Kohler, 1982; Lamsal, Koegel, & Gunasekaran, 2007; Nieuwland et al., 2021; Nissen et al., 2021; Tamayo Tenorio, Gieteling, de Jong, Boom, & van der Goot, 2016). The latter contributing largely to a potentially high usefulness in food products demanding stabilization of foams or emulsions.

Protein stabilized foams provide structure and texture to a range of food products, including meringues and mousses. Currently, mostly animal-based proteins, *i.e.*, egg white, are used as foam stabilizing agents in such foods. In general, the formation and stability of a food foam is dictated by the properties of the surfactant molecules used. Amphiphilic proteins dissolved in the aqueous phase are important food surfactants (Dickinson, 1999). Proteins in the continuous water phase

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will spontaneously diffuse to the air-water (AW) interface, where they adsorb due to interactions between hydrophobic patches on the protein surface and the interface. Adsorption of proteins results in a decrease in surface tension, which can be assessed as function of time. This provides insight in the interactions between protein and interface, and between different protein molecules. The rate of adsorption and protein reorganization at the interface affects the rate of the surface tension reduction (Graham & Phillips, 1979). Protein unfolding and adsorption at the interface are key aspects of foam stabilization (Murray, 2007) and are affected by pH, temperature and surface coverage (Dickinson, 1999). The surface charges of the proteins affect the attraction/repulsion of the molecules to the interface and to each other. The charges are in turn affected by the pH and presence of ions in the solution. In addition to adsorption related phenomena, *i.e.*, the reduction of surface tension, adsorbed proteins stabilize the interface by steric and electrostatic repulsive effects and by forming a viscoelastic interfacial film through intermolecular interactions. An indication of the stability of a protein-stabilized foam bubble can be given by the viscoelastic properties of the protein film (Murray, 2020). The viscoelastic properties of adsorbed protein films can be assessed by dilatational surface rheology measurements, such as the oscillating pendant drop method. Dilatational surface rheology provides information on protein-protein interactions at the interface and the capability of the adsorbed protein film to deal with dilatational deformations, such as those that might occur in a real food foam system (Graham & Phillips, 1980; Wierenga & Gruppen, 2010).

When using plant-based protein concentrates as ingredients in food products, the protein behavior at different pH values affects their applicability to a large extent. The solubility of the proteins is generally reduced in proximity of the protein isoelectric point (pI), the pH where the net charge of the protein is zero (Bhatia & Dahiya, 2015). At such pH values, protein aggregation can be expected, as repulsive effects between the protein molecules are diminished. In many food applications, *i.e.*, in foam stabilization, the solubility of proteins is an important prerequisite for them to diffuse to and adsorb at the interface (Damodaran, 2005).

The AW interface stabilizing properties of leaf proteins have been studied by others showing high foam capacity and foam stability of proteins extracted from, amongst others, lucerne (also known as alfalfa, *Medicago sativa*) (Knuckles & Kohler, 1982; Lamsal et al., 2007; Nissen et al., 2021; Sheen, 1991; Wang & Kinsella, 1976), sugarbeet (*Beta vulgaris*) leaves (Martin, Castellani, de Jong, Bovetto, & Schmitt, 2018), broccoli (*Brassica oleracea* var. *italica*), cabbage (*Brassica oleracea* var. *capitata*), cauliflower (*Brassica oleracea* var. *botrytis*), and beetroot (*B. vulgaris*) leaves (Sedlar, Ćakarević, Tomić, & Popović, 2021). Comparable, or better, foam stabilizing properties have been reported for LPCs from several green biomass sources as compared to commercially used plant proteins (*e.g.,* soy proteins) indicating the general suitability of LPCs for food applications (Martin et al., 2018). However, basic insights into the AW interfacial properties of LPCs are currently lacking. This remains a hurdle for their successful application in food.

When producing LPC for food applications in a biorefinery context, it is of the utmost importance that a wide range of green biomass can be used as raw material for the fractionation (Nynäs et al., 2021). Based on the hypothesis that LPCs from various green biomass sources are suitable as food ingredients in, *e.g.*, vegan food, with similar properties independent of source, we focused on the evaluation of solubility and aggregation propensity at a range of pH values as well as on AW interfacial properties of LPCs at neutral pH.

Thus, the aim of the present study was to evaluate the properties in relation to food applications of LPC from six different leafy biomass types, *i.e.*, lucerne, sugarbeet and beetroot leaves, mangold (*Beta vulgaris*), spinach (*Spinacia oleracea*), and kale (*Brassica oleracea* var. *sabellica*). The protein composition, protein solubility, foamability, and interfacial properties were studied and compared to the properties of commercially relevant egg white protein, which allowed evaluating the potential food applicability of these six LPCs.

2. Materials and methods

2.1. Extraction of leaf proteins

Green biomass from five different sources (lucerne, sugarbeet and beetroot leaves, mangold, and kale) was hand harvested from fields in the South of Sweden right before or after harvest of the main product and stored at −20 °C until further processing. Fresh baby spinach was purchased from a supermarket and frozen before processing.

The green biomass sources were fractionated to obtain the LPCs according to our previous work with protein extraction yields of approximately 0.5–2.0% (Nynäs et al., 2021). Briefly, thawed but still cold leaves were fed through a kitchen model juicer (Angelia 5500, Angel co. Ltd., South Korea) to extract the green juice (GJ) containing the water soluble proteins, including RuBisCO. The green color and cell debris were removed by heating the GJ to 55 °C followed by centrifugation (3200 RCF, 4 °C, 10 min). The clear yellow to brown supernatants contained the majority of the RuBisCO. The pH of the supernatant was adjusted to 4.5 by addition of 1 M HCl to facilitate precipitation of the proteins, and the proteins were separated by centrifugation (1150 RCF, 4 °C, 10 min). The clear supernatant was discarded and the protein precipitate was washed twice by dispersing the pellet in MilliQ water followed by centrifugation. The pH was kept at 4.5 during the washing. The precipitate was redispersed in MilliQ water and the pH was neutralized by dropwise addition of 0.1 M NaOH. After mixing at room temperature for 30 min the samples were centrifuged (260 RCF, 4 °C, 5 min) and the redissolved proteins in the supernatant were transferred to new containers. Water was added to the pellets, followed by 10 min of mixing and centrifugation as before. The supernatant was pooled with the previous one and the protein solution was lyophilized. The resulting product is referred to as leaf protein concentrate (LPC).

2.2. LPC composition

The protein (Nx6.25) content of the LPCs was analyzed with an automated 1108 Elemental Analyser (Thermo Scientific, Waltham, MA, USA). The presence of RuBisCO and other proteins in the LPC prior to lyophilization was verified by SDS-PAGE analysis using gradient precast minigels (Invitrogen Novex Bolt™, 4–12%, Bis-Tris Plus, ThermoFisher, Waltham, MA, USA), together with a prestained protein ladder (Invitrogen SeeBlue®).

The lipid content in the LPCs was determined by methylation of the lipids followed by quantification of the fatty acid methyl esters (FAMEs). For methylation of lipids 2 ml of 2% H_2SO_4 in MeOH was added to 30 mg of sample in triplicate, followed by heating in a heating block at 90 °C for 60 min. To enable quantification of FAMEs, a 17:0-methylester internal standard was added. The FAMEs were extracted by adding 1 ml of heptane (GC quality) and 1 ml of MilliQ water, followed by thorough mixing. The heptane phase containing the FAMEs was separated by centrifugation (1300 RCF, 21 °C, 2 min). For analysis an Intuvo 9000 GC (Agilent, Santa Clara, CA, United states) in combination with a 5977 MSD system, and a 7693 automatic liquid sampler (Agilent), were used. The Masshunter software (Agilent) was used for FAME identification and quantification. For separation, aliquots of 1.0 μl were injected to an Intuvo DB-23 column (30.0 m long, 0.25 mm in diameter, and a film thickness of 0.25 μm, Agilent) with helium as carrier gas at a constant flow of 2.5 ml/min, inlet flow temperature 250 °C, guard chip temperature 175 °C. The initial column temperature was held at 150 °C for 0.2 min, increased to 210 °C by 4 °C/min, and to 230 °C by 10 °C/min with a hold time of 7 min. A 1:1 split was used to separate the flow to the FID and MS detectors. Temperatures of the transfer line, ion source, and quadrupole were 250 °C, 230 °C, and 150 °C, respectively. Data was recorded at 40–550 amu with a solvent delay of 3 min.

2.3. LPC solubility

The solubility of the LPCs was assessed in triplicate at three different pH values (3.0, 5.0, and 7.0). Lyophilized protein extracts were mixed with MilliQ water to reach a concentration of 2.0 mg protein/ml. The pH of the solutions were adjusted to 7.0, 5.0, or 3.0, by dropwise addition of 1.0 M HCl. Each sample was divided into three aliquots, one of which was used for determination of the solubility in water at a given pH. To the two others, extraction buffers were added in 1:1 v:v proportion: i) Sodium phosphate buffer (0.10 M $Na_2HPO_4{\cdot}12H_2O$, 0.10 M $NaH_2PO_4{\cdot}2H_2O$, 4% sodium dodecyl sulphate (SDS)) to establish a solubility profile under non-reducing conditions and ii) sodium phosphate buffer with 4.0% SDS, 2.0% dithiothreitol (DDT), and 4 M urea to establish a solubility profile under reducing conditions. The samples were mixed by shaking at room temperature during 1 h, and the dissolved proteins were separated from the insolubles by centrifugation (10 000 RCF, room temperature, 10 min). The supernatants were filtered with a 0.45 μm syringe filter to remove any remaining particles and transferred to vials. The samples in buffer ii) were treated under nitrogen atmosphere. Buffer corresponding to that in i) was added to the water extracted supernatants at pH 3.0, 5.0, and 7.0 after removal of insoluble particles to ensure the same dilution factor for all samples.

The samples were injected (20 μl) on a BioSepS4000 size exclusion column (Phenomenex, Torrance, CA, USA) and analyzed using a SIL-HTc modular LC-2010 system (Shimadzu, Kyoto, Japan) using a sodium phosphate buffer with a 0.050 M $Na_2HPO_4{\cdot}12H_2O$, 0.050 M $NaH_2PO_4{\cdot}2H_2O$ solution containing 2.0% SDS as elution buffer and with a flow rate of 1.0 ml/min. The total runtime was 30 min and detection took place at 214 nm. The column temperature was set to 30 °C. Proteins of known sizes were used as apparent molecular mass markers: bovine serum albumin (66 kDa), chicken egg ovalbumin (43 kDa), lysozyme (14 kDa).

Reducing conditions were used to ensure that all proteins had dissolved, and the area under such profile could be designated as the total protein solubility (100%). However, as the background peak of DTT overlapped with the later part of the chromatogram (where small proteins eluted), the total soluble protein was calculated based on a combination of the chromatograms obtained under reducing and non-reducing conditions. Hence the relative solubility of the proteins in water was calculated using:

$$Solubility = \frac{Area_{total}}{Area_{peak\ group\ A} + Area_{peak\ group\ B}} \times 100$$

where $Area_{total}$ is the total peak area for a given sample in water at pH 3.0, 5.0, and 7.0 compared to the area of the first part ("Peak group A″) of the chromatogram for the corresponding sample analyzed under reducing conditions (buffer ii) combined with the area for the later part ("Peak group B″) of the chromatogram for the same sample analyzed under non-reducing conditions (buffer i). Fig. 1 illustrates an example.

2.4. Foaming properties of LPC

As a preliminary experiment, prior to investigating AW interfacial properties in more detail (see 2.6), the ability of the LPCs to form and stabilize foams was confirmed by a simplified foaming test. Solutions with protein concentrations of 4.0 mg soluble protein/ml were prepared from kale, sugarbeet, and lucerne LPC. To achieve this, LPC powder was mixed with MilliQ water and insoluble particles were removed by centrifugation (3200 RCF, 21 °C, 2 min). The pH of the solutions was not adjusted and was at ~7 for all samples. Air was introduced into the solutions during 60 s of whipping in graduated glass cylinders using a kitchen model milk frother (Rubicson®, Kjell&Company, Sweden).

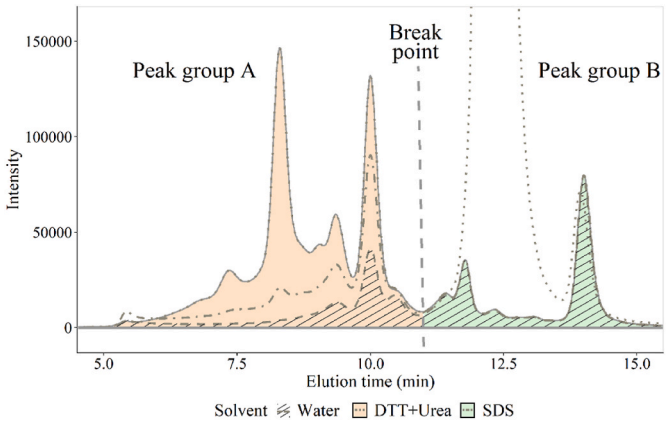

Fig. 1. An example (Spinach leaf protein concentrate (LPC) at pH 7.0) of how the LPC solubility was calculated. The large dotted peak at ~12.5 min is that of DTT.

2.5. Zeta potential and particle size measurements

The particle size distributions and the zeta potential (ZP) of different LPC dispersions were measured with dynamic light scattering and laser Doppler electrophoresis in a Zetasizer Nano ZS (Malvern Instruments, Malvern, United Kingdom) coupled to an MPT-2 autotitration unit (Malvern). For all calculations, the Malvern Zetasizer software was used.

Samples of each LPC were mixed with MilliQ water to reach a protein concentration of 1.0 mg protein/ml, corresponding to the "mid" concentration. The suspensions were shaken for 1 h at RT, filtered through 0.45 μm syringe filters and pumped into the autotitration unit and a disposable capillary zeta cell (Malvern). Readouts were made for both ZP and size distribution with titration intervals of 0.5 pH units. Solutions of HCl and NaOH (1.0 M and 0.1 M) were used for automatic adjustments of the pH while the sample was continuously circulated to ensure homogeneous mixing and pH adjustment. For measurements of the ZP of the LPCs in water without pH-adjustment (pH 7.0 ± 0.2), 1.0 ml of sample, prepared as those for titration, was transferred directly to disposable capillary zeta cells. All measurements were conducted in duplicates at room temperature.

2.6. (Oscillating) pendant drop analysis

Changes in surface tension (γ) of AW interface due to LPC constituent adsorption and rearrangement at the interface, as well as the dilatational interfacial rheological properties of the interface, were analyzed using a Theta optical tensiometer (Attension, Biolin Scientific, Stockholm, Sweden). Sample solutions at three different protein concentrations (Table 3), where the lowest concentration was a 10-fold dilution of the highest, and the mid concentration corresponded to 1.0 mg of total protein/ml, were prepared by mixing protein extracts with MilliQ water. After 1 h of mixing at room temperature the solutions were filtered through 0.45 μm syringe filters. The unadjusted pH values for the solutions were 7.0 ± 0.2. A protein extract of lyophilized egg white (Pulviver, Bastogne, France), with a protein content of 84.2% and a solubility of 88% in water at pH 7.0, was used as a reference.

A pendant drop with a fixed volume of 8.0 μl was created and the γ was monitored over a timeframe of 600 s, while recording one image per 7 s. The γ calculations were done by drop shape analysis and a Young-Laplace fit using the OneAttension software (Biolin Scientific, Stockholm, Sweden). Oscillatory measurements were initiated immediately after the pendant drop measurements, applying sinusoidal oscillations of the drop volume with an amplitude of 0.4 μl (which was within the linear range) and with a frequency of 0.5 Hz. During oscillatory measurements 7 frames per second were recorded for 10 cycles and drop shape analysis was performed as above. The surface dilatational

modulus E, which is the change in γ in relation to the relative change in the surface area (A), was determined based on the drop shape analysis made during the oscillations according to:

$$E = \frac{d\gamma}{dlnA}$$

The surface dilatational modulus E can be divided into a real surface dilatational elastic component (E') and an imaginary dilatational viscous component (E″), where E″ is defined by the surface dilatational viscosity (η_d), and the frequency (ω) of the variation in A (Lucassen--Reynders, 1993; Lucassen-Reynders, Benjamins, & Fainerman, 2010):

$$E = E' + iE'' = E' + i\omega\eta_d$$

The measurements were conducted at ambient temperature (18 °C ± 1 °C). Between each measurement the equipment was thoroughly cleaned and the surface tension of pure water was verified to be 72.0 ± 0.5 mN/m. Duplicate measurements were performed for two separate sample preparations for each crop and protein concentration.

2.7. Data treatment and statistical analysis

A general linear model analysis of variance (ANOVA) with Kenward-Roger's method and a significance level of $p < 0.05$, followed by Tukey post-hoc test, was used for the statistical analyses. The analyses were performed in RStudio version 1.4.1106 (RStudio Team, 2020), applying the function packages lme4, car, emmeans, lmerTest, multcomp, and multcompView.

Outliers due to errors in the pendant drop measurements were defined as data points for which the standard deviation as compared with its neighbors (*n-1*, *n+1*) exceeded 3.5, an arbitrarily chosen value that did not exclude other data points than those reasonably considered outliers. The outliers were replaced with the median value of the neighboring data points to keep the data sets complete and balanced. The lag time for the pendant drop measurements were arbitrarily set to the point where $\gamma < 69$ mN/m.

The rate of diffusion to and adsorption at the AW interface is given as the slope of the logarithmic regression of γ as a function of time. As equilibrium surface tension values were not reached during pendant drop analyses, an estimated equilibrium surface tension (EEST) was determined by linear regression with γ as a function of $1/\sqrt{t}$ for the linear part of the data, which was arbitrarily set to all data points with $1/\sqrt{t} < 0.075$. The EEST is the resulting γ when $t \to \infty$, and in practice the intercept of the linear regression.

3. Results and discussion

3.1. The leaf protein concentrates (LPC)

3.1.1. Composition

The leaf protein concentrates (LPCs) had a protein content (Nx6.25) ranging from 67.2% to 87.7% (Table 1). A lipid content ranging from 0.07% to 1.04% was found for the LPCs (Table 1), with the highest values (~1%) seen for spinach and sugarbeet.

Similar to previous studies (Hojilla-Evangelista et al., 2016; Sedlar et al., 2021; Tanambell, Møller, Corredig, & Dalsgaard, 2021), RuBisCO was the major protein present. Here represented on SDS-PAGE (Fig. 2a) as the two most intense bands at ~55 kDa and ~14 kDa, which are known to be the large subunit (LS) and small subunit (SS) (Andersson & Backlund, 2008). The high presence of RuBisCO was further verified by SE-HPLC with the most prominent peaks in the chromatograms found at elution times corresponding to those of molecules of similar sizes as the RuBisCO subunits (Fig. 2b, Fig. S1).

3.1.2. Solubility and aggregation behavior at different pH values

Protein solubilities were here determined by comparing areas under SE-HPLC curves of protein extracts at varying pH values to a reference profile, in which all protein was considered soluble. An advantage of this method is that, in addition to estimating the overall protein solubility, the molecular mass distribution of the proteins in the extracts can be assessed at the same time. Large variations in LPC protein solubility at pH values (7.0, 5.0, 3.0) relevant for food applications, were observed for LPCs from all evaluated biomass types (Table 2, Fig. 2b, Fig. S1). Sugarbeet LPC showed both the overall highest solubility of 67.6% at pH 7.0 and the overall lowest solubility of 9.2% at pH 5.0 (Table 2). In general, the highest solubility for the different LPCs was seen at pH 7.0. The difference between the solubility values at pH 7.0 on the one hand and at both acidic pH values on the other hand was significant ($p < 0.05$), while no significance was found between solubility values at pH 5.0 and 3.0. For some of the biomass types, *e.g.*, lucerne and mangold, the solubility was lower at pH 3.0 than at pH 5.0, while the opposite was observed for beetroot and sugarbeet.

The peaks at early elution times of the SE-HPLC chromatogram indicated a presence of large protein aggregates in many of the samples (Fig. 2b, Fig. S1). From the example of beetroot (Fig. 2b), it is clear that such peaks are present in profiles from samples dissolved in the non-reducing and reducing buffers, but in water, they were present at pH 7, but not at pH 3 and 5. At the latter pH values, these larger aggregates were likely rendered insoluble altogether, and would have been removed already when filtering the samples with 0.45 μm syringe filters prior to the analysis. The presence of peaks at low elution times, *i.e.,* at large apparent molecular weights, is in several cases linked to less prominent peaks for the RuBisCO subunits, especially the LS, which supports the presence of aggregates.

Previous studies have shown that a high LPC solubility is obtained at neutral and alkaline pH, while the solubility is low at pH 3.0 to 5.0 (Ducrocq et al., 2022; Lamsal et al., 2007; Martin et al., 2018; Nieuwland et al., 2021; Sheen & Sheen, 1985). Others have reported higher solubility values at pH 7.0, *e.g.*, 90% solubility of sugarbeet LPC (Martin et al., 2018), and 97% for lucerne LPC (Lamsal et al., 2007). The latter observation, however, was prior to lyophilization, which may affect the protein solubility. When comparing LPC solubility results from different studies, it is important to keep in mind that the solubility, even for similar biomass types, to a large extent depends on the processing history of the proteins, as the treatment effects formation and interruption of protein interactions (Lamsal et al., 2007; Nissen et al., 2021; Tanambell et al., 2021). Another important factor is the purity of the LPCs, as other phytochemical compounds, *e.g.*, polyphenolic compounds may associate strongly to the leaf proteins and reduce the solubility of the protein concentrates (Amer, Juul, Møller, Møller, & Dalsgaard, 2020). Finally, the conditions used for determination of the protein solubility, including the protein concentration tested and the centrifugation speed applied, might also explain differences between different

Table 1
Nitrogen and protein content (Nx6.25 on dry matter basis, n = 2) and lipid content (% FAME on dry matter basis, n = 3) for leaf protein concentrates from different biomass sources and for the reference protein egg white.

Biomass source		Nitrogen (%)	Protein (%)	Lipid (%)
Beetroot	*Beta vulgaris*, subsp. *vulgaris*, var. *Red hawk*	14.1 ± 0.1	87.8 ± 0.6	0.07 ± 0.01
Kale	*Brassica oleracea*, var. *sabellica*	11.6 ± 0.0	72.2 ± 0.2	0.15 ± 0.04
Lucerne	*Medicago sativa*	10.8 ± 0.0	67.2 ± 0.1	0.06 ± 0.01
Mangold	*B. vulgaris*, subsp. *vulgaris*, var. *cicla*	13.4 ± 0.0	83.4 ± 0.1	0.40 ± 0.00
Spinach	*Spinacia oleracea*	12.9 ± 0.1	80.8 ± 0.6	1.04 ± 0.29
Sugarbeet	*B. vulgaris*, subsp. *vulgaris*, var. *Lombok*	12.2 ± 0.1	75.9 ± 0.5	0.94 ± 0.02
Egg white			84.2[a]	n.d.

[a] Value provided by manufacturer, n. d.: Not determined.

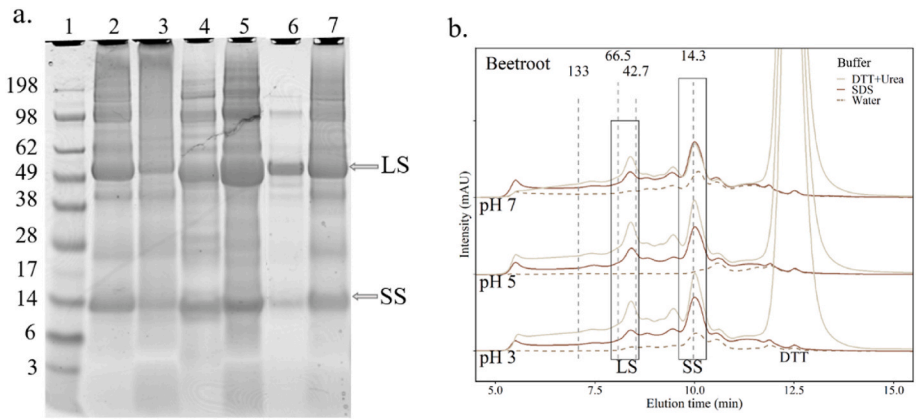

Fig. 2. a) SDS-PAGE analysis of leaf protein concentrates (LPCs) from the different biomass sources in water solutions at pH 7.0 prior to lyophilization. 1. Protein ladder (kDa), 2. Beetroot leaves, 3. Sugarbeet leaves, 4. Lucerne, 5. Spinach, 6. Kale, 7. Mangold. The large (LS) and small subunit (SS) of RuBisCO (~55 kDa and ~14 kDa) are marked with arrows. b) SE-HPLC chromatogram of beetroot LPC in different buffer solutions at different pH. The dashed vertical lines indicate the elution times of proteins of known size (kDa).

Table 2
Isoelectric point (pI) and solubility in water (%) at different pH values at ambient temperature (~21 °C) for leaf protein concentrates from different biomass sources. The letters indicate results not significantly different from each other within each column.

Biomass source	pI	pH 3	pH 5	pH 7
Beetroot	4.0 ± 0.0 ^{c}	16.9 ± 3.5 ^{b}	13.3 ± 4.1 ^{ab}	46.5 ± 1.4 ^{ab}
Kale	3.6 ± 0.0 ^{a}	27.4 ± 8.1 ^{a}	26.7 ± 5.4 ^{a}	54.1 ± 4.6 ^{b}
Lucerne	3.7 ± 0.1 ^{ab}	12.6 ± 0.6 ^{a}	35.2 ± 3.2 ^{a}	63.4 ± 11.6 ^{ab}
Mangold	3.8 ± 0.1 ^{b}	12.4 ± 4.7 ^{a}	46.4 ± 20.7 ^{b}	50.1 ± 11.9 ^{ab}
Spinach	3.8 ± 0.1 ^{b}	12.9 ± 5.0 ^{a}	18.2 ± 2.9 ^{ab}	41.0 ± 1.0 ^{ab}
Sugarbeet	3.8 ± 0.0 ^{b}	16.7 ± 0.8 ^{a}	9.2 ± 4.0 ^{a}	67.6 ± 7.9 ^{a}

studies.

Through particle size and ZP measurements during titration of LPC dispersions, we revealed an aggregation pattern of the proteins for the LPCs (Fig. 3), which is well in line with the solubility data presented in Table 2. A minimum solubility is normally found in the vicinity of the pI of the LPC. For the six LPCs evaluated here, the low solubility values in the pH region of 3.0–5.0 indeed correspond well to the pI values of 3.6–4.0 that were observed for the different LPCs (Table 2, Fig. 3), which also correspond to the previously reported pI value for lettuce of 3.8 (Ducrocq et al., 2022). Moreover, pronounced aggregation in LPC dispersions, as identified here by a drastic increase in the average particle size, was observed in the pH range of 3.0–5.0 as well (Fig. 3). The largest average particle sizes were observed at, or in proximity to, the pI. The limit for the particle size analysis with the current instrument is 10 μm according to the manufacturer, and all exceeding values should be interpreted with care, but the general aggregation pattern for the LPCs is nonetheless obvious. It is of importance to remember that the LPCs are not pure RuBisCO isolates. Hence the measured pI is that of the complex LPC dispersion, including other proteins as well as other compounds (*i.e.*, various phytochemicals and salts co-extracted with the protein), explaining the discrepancy between the results obtained here and, *e.g.*, the pI that has been calculated for pure spinach RuBisCO at pH 6.03 (Nynäs et al., 2021). The size distributions (results not presented) indicated particles of varying size in the sample dispersions. Such polydispersity might well affect the interfacial behavior, but studies in more detail would be needed to understand these correlations, as this does not fall within the scope of the present article.

3.2. *Preliminary study on LPC foaming capacity*

By a simplified foaming test we conclude that a relatively stable foam (>50% of the foam was still intact after 15 min for all samples, results

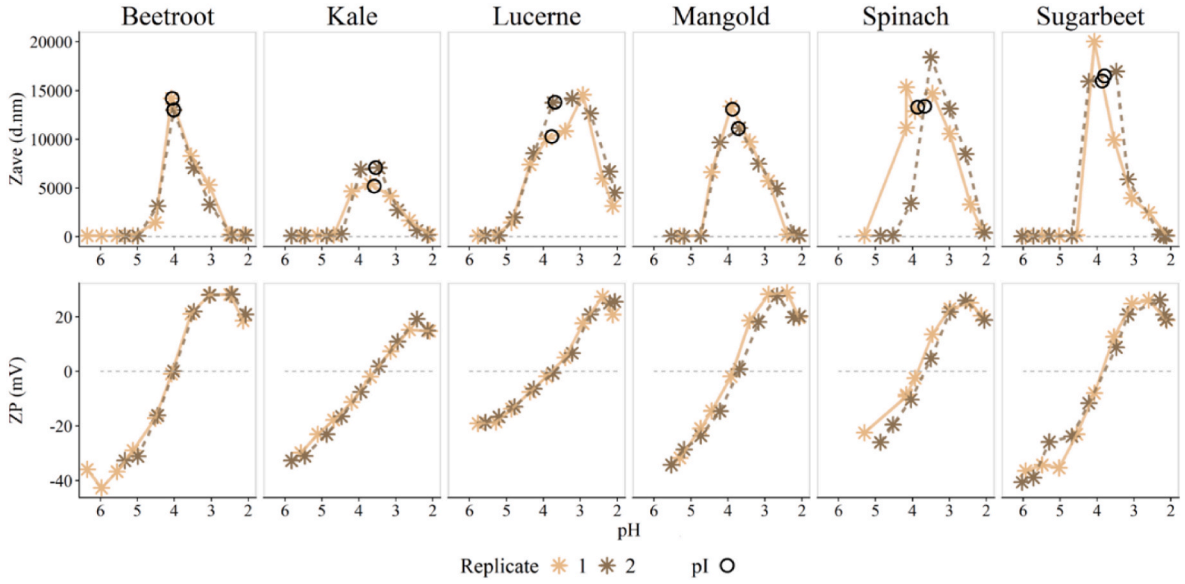

Fig. 3. The average particle size (Zave) and zeta potential (ZP) during titration at ambient temperature (~21 °C) for leaf protein concentrates (LPCs) from different biomass sources in water (1 mg protein/ml). The different colors represents the replicates and the circles mark the pI of the LPC solutions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

not shown) was formed when introducing air into the evaluated LPC solutions (sugarbeet, kale, and lucerne) by whipping (Fig. 4) as has also been reported in previous studies (*e.g.,* Lamsal et al., 2007; Sedlar et al., 2021). Others have reported LPC foaming capacity equal to, or higher than, egg white (Knuckles & Kohler, 1982; Sheen & Sheen, 1985) and whey protein (Martin et al., 2018). From this study, it was clear that the foaming capacity, *i.e.*, the volume of foam produced, varied somewhat between the LPCs. No numeric results were recorded in the qualitative test, but in Fig. 4 the upper limits of the foams are marked, indicating approximate foam volumes of 15 ml for lucerne and kale LPCs, while the approximate volume for sugarbeet is considerably lower at around 10 ml. Based on both our present results and those of others, it can be assumed that the LPCs from the other biomass types included in this study would form similar foams as sugarbeet, kale, and lucerne. Here we opted not to focus further on the foaming properties of the different LPCs, since others already have reported such results, but performed an in-depth investigation into their AW interfacial properties.

3.3. Air-water interfacial properties of LPCs

3.3.1. Dynamic surface tension measurements

The evolution of surface tension (γ) over time was assessed with optical tensiometry experiments at pH 7.0. For all LPCs and egg white, at all the evaluated protein concentrations, a decrease in γ was observed over the 10 min time frame of the pendant drop experiment. The evolution of γ over time, which relates to the rate of diffusion to and adsorption at the AW interface of LPC constituents, was clearly similar among the LPCs from the different biomass types and also between the LPCs and egg white protein used as reference. (Fig. 5, Table 3). For the lowest protein concentration ("low") assessed, a lag phase was seen for all LPCs, as more time is needed for sufficient protein to diffuse to, and adsorb at, the AW interface. No statistically significant differences were seen among the protein sources with regard to the lag time (Table 3). Generally, a logarithmic (base 10) regression showed a good fit to the data, especially when taking the lag phase into consideration. The slope can be considered as a measure of the overall rate of adsorption and continued diffusion to the interface (Table 3). The protein concentration had a statistically significant effect on the slope of γ as a function of time ($p < 10^{-7}$), but the effect of the protein source was small. As the solubility at pH 5.0 and 3.0 was low and a certain level of solubility is a prerequisite for LPCs to be used as proper interface stabilizing agents, the tensiometric measurements were only conducted at pH 7.0. Performing tensiometry measurements at different LPC concentrations provides information on possible differences in diffusion and adsorption behavior of the LPCs.

At the "mid" concentration for kale and lucerne LPC (0.5 and 0.6 mg soluble protein/ml, respectively), sufficient protein was available to ensure optimal adsorption and surface coverage at the AW interface, as the curves of γ as a function of time for the "mid" and "high" concentration were very similar (Fig. 5). This was not the case for the other protein sources, even though higher protein concentrations were used. Although, the results of the pendant drop measurements are in line with corresponding experimental results for other plant proteins, *e.g.*, wheat gluten hydrolysates (Wouters et al., 2017) and with general theories on protein behavior at AW interfaces (Graham & Phillips, 1979; Wierenga & Gruppen, 2010), comparisons of LPCs from various sources have not been evaluated previously. The kinetics of diffusion and adsorption of a protein at a given concentration depend on its molecular mass, charge, surface hydrophobicity and molecular flexibility (Graham & Phillips, 1979; Murray, 2007; Wierenga & Gruppen, 2010). Thus, the similarity of the results for all the LPCs illustrates that no major changes in protein structural properties, and thus of their surface activity, seem to occur for LPCs of different origin. This underpins the hypothesis that green biomass of different origin can be used in a biorefinery concept to produce LPCs of similar quality.

The duration of the pendant drop experiment was not sufficiently long to reach equilibrium γ values. Therefore, an EEST (estimated equilibrium surface tension) was determined as outlined in section 2.7 (Table 3). EEST values were around 50 mN/m for most of the samples, except for the lowest concentration of kale LPC (61.9 mN/m) and for the highest concentration of sugarbeet LPC (46.8 mN/m). For pure protein systems an equilibrium γ around 45–50 mN/m is expected (Bos & Van Vliet, 2001). That EEST values for the mid and high concentrations were in many cases similar again indicates that at the mid concentration, sufficient protein material was present for complete surface coverage.

3.3.2. Dilatational rheology measurements

All the LPC protein films at the AW interface showed a viscoelastic character, with considerably larger elastic contributions (E') than viscous contributions (E″) (Fig. 5 bottom, Table 3). This is a common feature for protein films formed at AW interfaces (Dickinson, 1999). The E values for all the LPCs were similar to that of the egg white protein, which is an excellent foam stabiliser. This indicates great potential for using LPC, from different biomass sources, as a plant based functional ingredient in foam applications.

The oscillating drop measurements were initiated immediately after the γ measurements, *i.e.*, 10 min after drop formation. Overall, for all biomass types together, E' and E″ values were not significantly affected by the protein concentration ($p > 0.05$, Table S2). However, the effect of protein concentration differed for the biomass types, with a positive correlation for lucerne, beetroot, and spinach LPC, and also for the reference protein egg white (Table 3). Such behavior can be expected in situations where more protein accumulates at the interface at increasing bulk concentrations, which might be the case for these LPCs. In contrast, for kale and mangold LPC the "mid" concentration had the highest E' and E″ values, which indicates that the interface might have already been saturated at this "mid" concentration. Finally, a negative correlation was obtained for sugarbeet LPC. These effects could be explained by

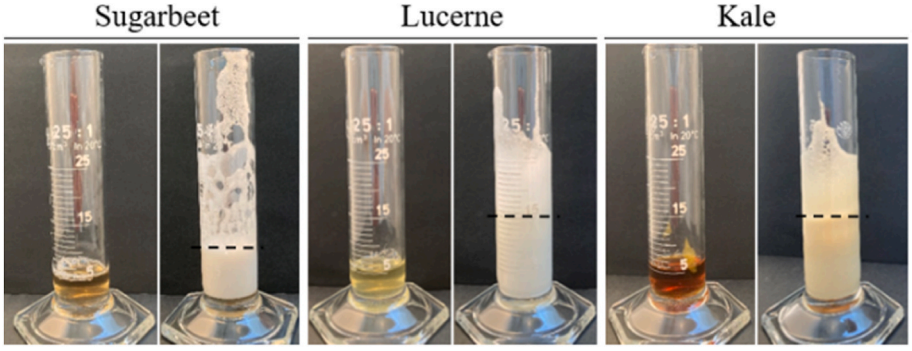

Fig. 4. Foams made of sugarbeet, lucerne, and kale leaf protein concentrates solutions at ambient temperature (~21 °C) with a concentration of 4.0 mg soluble protein/ml and unadjusted pH (~7). The dashed line represents an approximation of the upper foam surface.

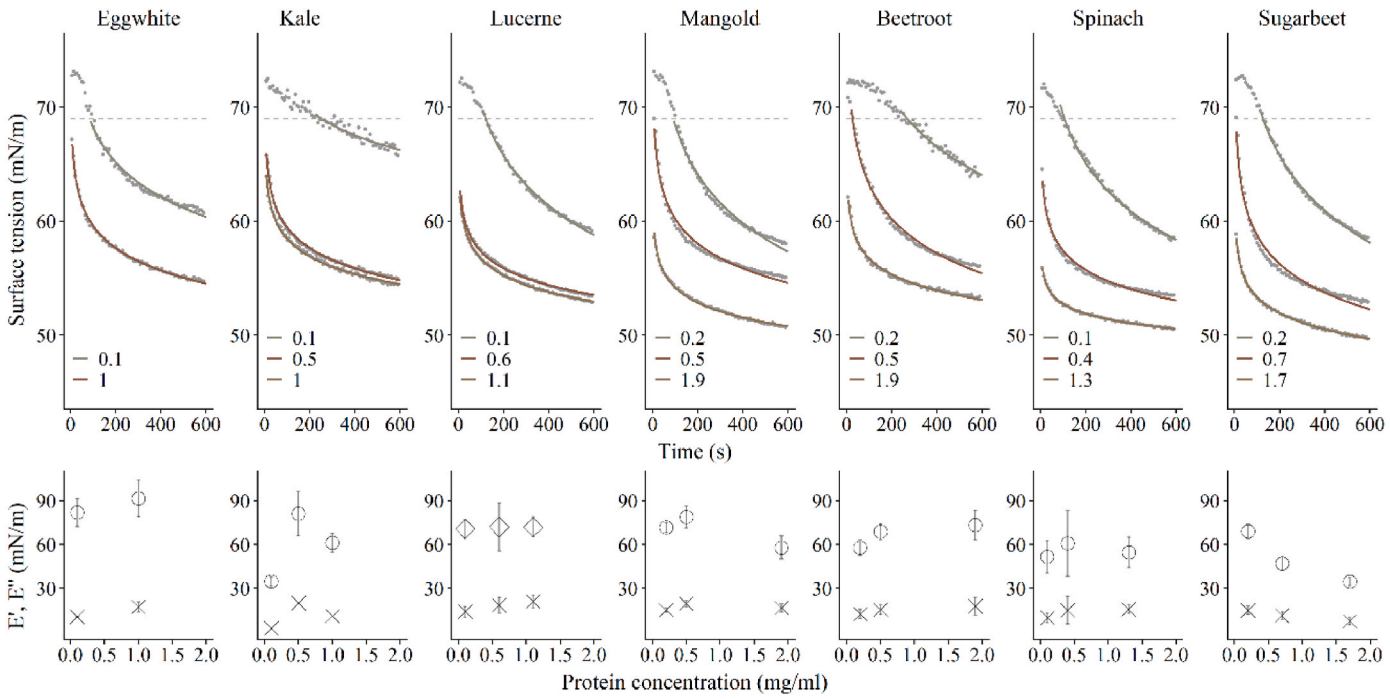

Fig. 5. Top: The average surface tension as function of time as measured with the pendant drop method at ambient temperature (18 °C) for leaf protein concentrate solutions at pH ~7. The colors indicate the protein concentration (mg soluble protein/ml) and the line is the logarithmic regression for the average values. The dashed line is the (arbitrarily drawn) lag phase limit at 69 mN/m, and no data points above that limit were included in the data regressions. Bottom: The E' (×) and E″ (O) for leaf protein concentrates from different biomass sources at different protein concentrations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the relatively high content (1.04% in sugarbeet LPC and 0.40% in mangold LPC) of potentially interfering lipids in the LPCs (Table 1). Indeed, lipids – even at low concentrations – might affect the ability of proteins to stabilize AW interfaces (Wilde, Mackie, Husband, Gunning, & Morris, 2004). As the protein bulk concentration increases, so does the lipid concentration, but given the more rapid diffusion and adsorption of lipids at the AW interface, their relative contribution in disrupting the protein film may increase with bulk concentration (Janssen, Wouters, & Delcour, 2021). However, the same effect was not observed for spinach LPC, which also contains ~1% lipids. We hypothesize that at even higher bulk concentrations, this effect would also be at play for the spinach LPC. In addition, the type and polarity and thus surface activity of lipid molecules present in the different LPCs may have been different, which would have to be investigated further. Other phytochemicals present in the LPCs were assumed to have little effect on the interfacial behavior and were not investigated in the present study, but for more in-depth studies, especially regarding nutritional aspects of LPCs, such compounds should be identified and quantified.

Although we here for the first time provided an in-depth analysis of the AW interfacial properties of LPC proteins, it remains to be investigated how these data relate to the foaming properties of LPCs. As it is well-known that foam stability of protein solutions depends on the strength of the interfacial film that is formed (Graham & Phillips, 1980; Kloek, Van Vliet, & Meinders, 2001; Murray, 2007), the here observed high E values in combination with the qualitative foam test (see section 3.2) are promising with regard to the ability of LPCs to potentially stabilize foam. Still, the exact mechanisms at play in LPC foam stabilization would have to be further investigated, as amongst others also steric and electrostatic effects of adsorbed proteins at gas bubbles can affect foam stability.

In this context, it is relevant that the net charge of the LPC solutions, *i.e.*, the ZP, in water at the initial pH (7.0 ± 0.2) was negative for all samples with values ranging from −33.4 to −19.2 mV (Table S3). There is too little data available to enable evaluation of the effect of ZP and particle size on the E' and E″ values. All the LPC solutions did have a negative net charge at the pH (~7) used in the measurements, which probably resulted in electrostatic repulsion. At pH values closer to the pI, the behavior would most likely be different, but the solubility would be hampered, which clearly was the case at pH 3 and 5 (Table 3). In future studies including foam stability measurements, pH values closer to the pI, especially that of RuBisCO (~6) should be included, as the minimized electrostatic repulsion should have a positive effect on the foam stability. Additionally the effect of temperature on the protein behavior would be of interest for closer studies, as elevated temperatures has resulted in higher foamability and foam stability for other plant proteins (Damodaran, 2005).

4. Conclusions

Many different leafy biomass sources are available to be used as raw material for producing LPC (Nynäs et al., 2021), a potential protein ingredient in foods. The foaming test and the pendant drop measurements performed in the present study indicated no major differences in interfacial properties among LPCs from different sources. This is advantageous especially for industrial LPC production, since a broad range of interchangeable biomass sources can be used. Opportunities to produce LPC with similar properties independent of the biomass used significantly prolongs the production season in a green biomass fractionation facility without interfering with the functional properties of the end product. It also opens up possibilities for the use of mixed leafy biomasses, which often may be the case, *e.g.*, if using ley grasses. Importantly, the interfacial behavior of the LPCs was very similar to that of egg white, a commonly used food foam stabilizing agent, which further strengthens the potential of LPCs to be used as a plant based functional food ingredients.

Further studies with an in-depth assessment of the foaming capacity as well as the foam stability at acidic pH values would increase the understanding of the LPC potential in a broader range of food system

Table 3
Results from the (oscillating) pendant drop measurements for leaf protein concentrates from different biomass sources and the reference protein, egg white, at different concentration levels, including the slope for the logarithmic (base 10) fit, the lag time, the estimated equilibrium surface tension (EEST), the viscous contribution E', and the elastic modulus E. All measurements were performed at ambient temperature (18 °C) and at pH ~7. The slope is from a logarithmic regression of the surface tension as function of time. The letters indicate values (column wise) not significantly different ($p < 0.5$) from each other.

Biomass source	Conc level	Conc (mg/ml)	Slope	Lag time (s)	EEST (mN/m)	E'	E
Beetroot	Low	0.2	-14.3 ± 1.6^{abc}	266.4 ± 116.7^{a}	56.3 ± 2.4^{cd}	57.9 ± 5.3^{abcde}	12.4 ± 3.2^{abc}
	Mid	0.5	-10.1 ± 1.9^{bcd}	–	50.7 ± 0.4^{abc}	68.9 ± 5.4^{bcde}	15.4 ± 3.2^{abc}
	High	1.9	-4.7 ± 0.6^{de}	–	50.7 ± 0.3^{abc}	73.3 ± 10.2^{cde}	17.7 ± 6.1^{bc}
Kale	Low	0.1	-9.0 ± 2.6^{bcd}	206.4 ± 149.7^{a}	61.9 ± 4.3^{d}	34.7 ± 3.1^{ab}	2.8 ± 0.9^{a}
	Mid	0.5	-5.9 ± 0.9^{de}	–	51.5 ± 0.3^{abc}	81.1 ± 15.0^{cde}	19.9 ± 5.2^{bc}
	High	1	-5.1 ± 0.5^{de}	–	50.9 ± 0.4^{abc}	61.2 ± 6.5^{abcde}	11.0 ± 2.9^{abc}
Lucerne	Low	0.1	-14.5 ± 3.8^{ab}	106.2 ± 32.4^{a}	49.9 ± 4.0^{abc}	70.9 ± 6.3^{cde}	14.0 ± 3.6^{abc}
	Mid	0.6	-4.9 ± 0.7^{de}	–	50.1 ± 1.3^{abc}	72.1 ± 16.5^{cde}	18.5 ± 5.4^{bc}
	High	1.1	-4.8 ± 0.4^{de}	–	49.8 ± 0.5^{abc}	72.0 ± 6.7^{cde}	20.9 ± 4.1^{c}
Mangold	Low	0.2	-14.4 ± 0.9^{ab}	93.6 ± 36.0^{a}	49.8 ± 0.8^{abc}	71.7 ± 3.3^{cde}	15.0 ± 1.5^{abc}
	Mid	0.5	-7.3 ± 1.1^{de}	–	51.6 ± 1.2^{abc}	78.9 ± 7.5^{cde}	19.5 ± 2.0^{bc}
	High	1.9	-4.4 ± 0.1^{de}	–	47.7 ± 1.0^{a}	57.9 ± 7.9^{abcde}	16.6 ± 2.7^{abc}
Spinach	Low	0.1	-14.9 ± 2.1^{ab}	106.2 ± 57.4^{a}	48.7 ± 2.1^{ab}	51.7 ± 11.1^{abcd}	9.9 ± 3.1^{abc}
	Mid	0.4	-5.6 ± 1.1^{de}	–	50.8 ± 0.9^{abc}	60.9 ± 22.5^{abcde}	15.0 ± 9.5^{abc}
	High	1.3	-2.8 ± 0.2^{e}	–	48.9 ± 2.6^{ab}	54.7 ± 10.4^{abcd}	15.7 ± 2.9^{abc}
Sugarbeet	Low	0.2	-16.2 ± 1.5^{a}	118.8 ± 34.0^{a}	48.4 ± 1.9^{a}	69.1 ± 4.9^{bcde}	14.8 ± 2.8^{abc}
	Mid	0.7	-8.3 ± 1.2^{cde}	–	49.2 ± 0.8^{ab}	47.1 ± 4.7^{abc}	11.3 ± 2.6^{abc}
	High	1.7	-4.7 ± 0.2^{de}	–	46.8 ± 0.6^{a}	34.5 ± 2.6^{a}	7.4 ± 2.5^{ab}
Egg white	Low	0.1	-10.0 ± 0.7^{bcd}	91.8 ± 29.6^{a}	55.2 ± 1.0^{bcd}	82.0 ± 9.6^{de}	10.4 ± 0.5^{abc}
	High	1	-6.5 ± 0.9^{de}	–	50.7 ± 0.9^{abc}	91.6 ± 12.7^{e}	17.3 ± 3.3^{bc}

relevant conditions. Notable in this regard is that we showed the solubility of the leaf proteins to be strongly affected by the pH, with very low solubility at pH 3.0 and 5.0, which is in line with the pI values ranging from 3.6 to 4.0 for LPCs from the evaluated biomass types. In addition, future studies should provide more in-depth foaming comparisons between LPC and animal proteins commonly used today, both in model systems and in actual food models (*e.g.*, meringue, cake, etc.), Finally, the functionality of LPC proteins in other applications, *e.g.*, gelling and emulsification, should be explored further.

Process optimization for extracting the proteins may affect the quality, and also the properties of the resulting protein concentrate. The LPCs are mixtures of different proteins present in the biomass, with RuBisCO as one of the major proteins. The range of different proteins present in the LPCs might also contribute to their foam stabilizing properties. With this in mind, a less selective extraction method where other leaf proteins are included in the final LPC, might be beneficial for increasing the extraction yields without losing any functional properties.

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CRediT authorship contribution statement

Anna-Lovisa Nynäs: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **William R. Newson:** Conceptualization, Methodology, Supervision, Validation, Writing – review & editing. **Maud Langton:** Supervision, Writing – review & editing. **Arno G.B. Wouters:** Conceptualization, Methodology, Project administration, Supervision, Validation, Writing – review & editing. **Eva Johansson:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors to the manuscript with the title: *Protein concentrates from various leaf sources as an alternative additive in vegan food: solubility at food-relevant* pH *values and air-water interfacial properties* all declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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