



# Aquatic weeds as novel protein sources: Alkaline extraction of tannin-rich *Azolla*



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## ABSTRACT

The aquatic weed *Azolla* is a potential protein crop due to its prolific growth and high protein content, supported entirely by nitrogen-fixing symbionts. Alkaline protein extraction at pH 8 followed by acid precipitation allowed recovery of 16–26% of the biomass nitrogen, while at pH 10.5 nitrogen recovery improved to 35–54%. This pH effect was typical of ferns of the family *Salviniaceae*, and may be explained by high concentrations of condensed tannins (CTs) in the biomass that precipitate protein at mild pH. Two approaches were tested to increase protein yield and reduce protein binding by CTs. Pre-extraction with aqueous acetone (70 v/v%) removed 76–85% of the CTs and subsequent alkaline extraction at pH 12.5 and 95 °C recovered 38% of the biomass nitrogen. Extraction with 1.5% of PEG as a CT-binding agent, also permitted to recover 38% of the nitrogen, under milder conditions of pH 8 and 45 °C.

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## 1. Introduction

The world population is expected to grow to more than 9 billion people in 2030 [1], which will pose a challenge for global food supply. Especially the amount of digestible protein is limiting in the diets of many population groups [2]. Currently soy production plays a major role in meeting demand for dietary protein, mostly indirectly by use as animal feed for livestock, which in turn provides animal protein for human consumption [3]. Soy production is concentrated in (sub)tropical regions of Brazil, USA and Argentina and imported in large quantities in temperate regions, such as western Europe [3]. Given the increasing demand for dietary protein and the geographically concentrated production of soy, novel sources of plant protein are sought, particularly for temperate regions.

*Azolla* is a genus of rapidly growing, nitrogen-fixing aquatic ferns that thrive in tropical to temperate regions of the world [4–6] *Azolla* biomass is particularly rich in protein with a favorable amino acid profile compared to soy [7,8]. Therefore it has been proposed as a novel protein source for feed [7,9–12] and, potentially, human

consumption [13]. *Azolla* biomass, however, also contains high amounts of (poly)phenols, which may (in part) explain the reduced digestibility observed in feeding trials when the *Azolla* fraction in the diet exceeds a threshold value between 5–15% depending on the animal [7,9–12]. Additionally, due to its high water content, long distance transport of *Azolla* biomass is environmentally and economically unsustainable, which restricts its consumption to a geographical area close to the cultivation site. One approach to broaden the application of *Azolla* biomass as a future protein source is to perform protein extraction locally and distribute the protein extract instead of the whole plant. Protein extracts have been successfully produced from seeds, microalgae, macroalgae, grasses and green tea leaves, using alkaline protein extraction [14]. Alkaline protein extraction was further used to produce protein extracts (6–7.2% nitrogen) from the duckweeds *Lemna*, *Spirodela* and *Wolffia* (extraction yields were not reported), but has not yet been tested on *Azolla* [15]. Fasakin (1999) used leaf pressing followed by heat congelation on *A. africana* to produce a high purity protein concentrate (11.4% nitrogen) containing 62.1% of the initial pulp nitrogen [16]. Although providing high yields, heat coagulation denatures protein whereas alkaline extraction can yield soluble protein, which broadens the scope for product applications. Therefore, in this study we investigate alkaline protein extraction for the production of protein from *Azolla* biomass of *Azolla*. Firstly we study protein yield from *Azolla* in relation to extraction pH and temperature. Secondly we apply alkaline extraction to a selection of aquatic weeds (*A. pinnata*, *S.*

Abbreviations: AAs, BSA; CT, N<sub>AA</sub>; PEG, PVPP; RT, THM, UV/VIS.

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*molesta* and *L. minor*), a macroalgae (*Ulva lactuca*) and soybean as a control (*Glycine max* seeds) to evaluate the effect of the biomass composition protein extractability. Finally we use the knowledge obtained from these experiments to test approaches for recovering protein from *Azolla* with a lower content of (poly)phenols.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

*A. filiculoides* was obtained from a location at Galgenwaard, Utrecht, the Netherlands. *A. pinnata* was obtained from the International Rice Research Institute (IRRI, Los Banos Phillipines), under accession number 535. *S. molesta* was obtained from the collection of the Utrecht University Botanical Garden. *A. filiculoides*, *A. pinnata* and *S. molesta* were grown in a growth chamber providing 16 h light at an intensity of  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ , a stable temperature of  $21^\circ\text{C}$  and 70% humidity. The growth medium for *Azolla* species consisted of 0.65 mM  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 1.02 mM  $\text{K}_2\text{SO}_4$ , 1 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 1.65 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 17.9  $\mu\text{M}$   $\text{Fe-EDTA}$ , 9.1  $\mu\text{M}$   $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 1.6  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 18.4  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 0.8  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.3  $\mu\text{M}$   $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.2  $\mu\text{M}$   $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ . To grow *S. molesta* the medium was supplemented with 2 mM  $\text{NH}_4\text{NO}_3$ . Medium pH was adjusted to 5.5 using KOH. Every two weeks the growth medium was replaced completely. One third of the biomass was harvested weekly and freeze-dried. Batches of freeze-dried biomass were pooled and used for laboratory scale protein extractions. Freeze-dried biomass of *Lemna minor* was provided by Adrie van der Werf (Wageningen University and Research, the Netherlands) the green seaweed *Ulva lactuca* was obtained from OceanHarvest (Galway, Ireland) and soybeans purchased at a local store.

For larger scale protein extractions, *A. filiculoides* was grown in a greenhouse of the Utrecht University Botanic Gardens from August 2016 to November 2016, without additional light or heating. Plants were grown in 12 l containers with a surface area of  $459 \text{ cm}^2$ . The growth medium was as described above and was replaced every two weeks. Harvested material was directly used for protein extraction.

### 2.2. Chemicals

NaOH, PEG (3350 & 6000 Da) and sodium-ascorbate ( $\geq 98\%$ ) were purchased from Sigma Aldrich. HCl, (38%) and anhydrous acetone (99%) were obtained from Merck. Tannic acid, chlorogenic acid, catechin and Bovine Serum Albumin (BSA), used for reference, were purchased from Sigma Aldrich. Black spruce tannins were provided by Caroline M. Preston [17] and characterized by Nierop et al. [18].

### 2.3. Protein extraction

For laboratory scale protein extraction 20 mg of freeze-dried *A. filiculoides* biomass was ground using a tissue-lyser. After grinding 1 ml of solvent was added and tubes were vortexed. The solvent consisted of water with different concentrations of NaOH and additives to test, such as poly ethylene glycol (PEG MW 3350 and 6000 Da) and sodium ascorbate. The pH was adjusted during the extraction to remain around 8.0, 10.5 and 12.5. The extraction was performed for 4 h at either room temperature (RT),  $45^\circ\text{C}$  or  $95^\circ\text{C}$ . Solubilized compounds were separated from the insoluble fraction by 10 min of centrifugation at  $6000 \times g$ . The protein precipitate was obtained by adjusting a known amount of the soluble fraction to pH  $3.5 \pm 0.25$  using HCl followed by 10 min of centrifugation at  $6000 \times g$ , at room temperature. The fractions, i.e. insoluble, soluble, protein precipitate and supernatant, were collected in pre-

weighed tubes, freeze-dried and weighed. The extraction was performed in nine technical replicates. Of these replicates, three were used for carbon and nitrogen (C and N) determination.

For larger scale extraction fresh *Azolla* biomass was surface dried on a filter paper, weighed and a aliquot was taken to determine the dry weight. Surface dried biomass and solvent were mixed in a blender at a 1:3 fresh weight: solvent ratio. After  $2 \times 1 \text{ min}$  of mixing the solution was transferred to 50 ml tubes, after which the procedure was followed as described above. Extractions were performed in technical triplicates.

### 2.4. Chemical analysis

For total C and N content, 1–2.5 mg of the freeze-dried material was analyzed using an elemental analyzer (Fisons NA 1500 CNS) connected to a mass spectrometer (Finnigan Delta Plus). Crude protein were derived from total N measurements by multiplying these with a factor 4.9, as was previously experimentally determined for *A. filiculoides* and *A. pinnata* [8]. Lipids were determined by extracting the freeze-dried biomass in a mixture of DCM:MeOH 9:1 in a soxhlet device and weighing the mass of the extract after evaporation of the solvent under vacuum and continuous nitrogen-flow. Total phenol was determined by extracting samples with aqueous acetone (70%) for 24 h at  $20^\circ\text{C}$  followed by reacting the samples with Folin-Ciocalteu reagents as described by Waterman (1994) [19].

For characterization of soluble and insoluble carbohydrates in *Azolla* biomass, first two successive Soxhlet extractions using water and ethanol were performed to remove non-structural components [20,21]. The extracted *Azolla* feedstock and aqueous extract were hydrolyzed in two steps: 12 M  $\text{H}_2\text{SO}_4$  at  $30^\circ\text{C}$  for 1 h, followed by 1.2 M  $\text{H}_2\text{SO}_4$  at  $100^\circ\text{C}$  for 3 h. The hydrolysate was analyzed using High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD, ICS3000, Dionex, Sunnyvale, CA) with a CarboPac PA1 column. Analysis was performed using an eluent sequence for lignocellulosic carbohydrates [20]. Carbohydrate determination in the starting material and insoluble residue after protein extraction was performed without pre-extractions and using an eluent sequence optimized for seaweed carbohydrates (i.e., including uronic acids). The ash content of *Azolla* feedstock was determined by combustion at  $550^\circ\text{C}$  in duplicate.

Thermally assisted Hydrolysis and Methylation (THM), i.e. pyrolysis in the presence of tetramethylammonium hydroxide (TMAH), was also employed to provide more insight into the polyphenolic composition. Prior to THM, samples were pressed onto Curie-point wires, a droplet of TMAH (25% in water) was added and samples were dried under a 100 W halogen lamp. Analysis of the THM products by way of GC-MS was identical to conventional pyrolysis. Compound identification was carried out by way of mass spectral comparison using a National Institute of Standards and Technology (NIST) library, interpretation of the spectra, retention times and/or comparison with literature data.

For detection of condensed tannins, the acid butanol assay was performed by adding to the freeze-dried sample 1.2 ml of *n*-butanol-HCl solution (5 v/v%) and 20  $\mu\text{l}$  of 2%  $\text{NH}_4\text{Fe}(\text{SO}_4)_2$  in 2 N HCl. Reagents were vortex mixed heated at  $95^\circ\text{C}$  for 50 min before measuring absorbance at 550nm [22]. Black spruce tannins were used as the reference standard.

### 2.5. Calculations

The mass balance of protein extractions was made by firstly correcting the mass of each freeze-dried fraction (insoluble, soluble, protein precipitate and supernatant) for the content of  $\text{Na}^+$ ,  $\text{Cl}^-$ , sodium ascorbate and/or PEG in the remaining solvent.

The mass of the soluble fraction, protein precipitate and supernatant was further corrected for the volume used for analysis, leading to the corrected mass ( $M_{fc}$ ) as following:

$$M_{fc} = \frac{(V_t/V_a) \cdot (M_f - V_{rf} \cdot C_s)}{M_{start}}$$

Where  $V_t$  = the total volume of the soluble fraction after centrifugation,  $V_a$  is the volume used for analysis,  $M_f$  = mass of the fraction after freeze-drying,  $V_{rf}$  = the residual volume of solvent left in the fraction before analysis,  $C_s$  = the mass concentration of the solvent,  $M_{start}$  = mass of the starting material after freeze-drying. In the cases that the soluble fraction and the supernatant were not analyzed,  $V_t = V_a$ . The corrected nitrogen balance ( $N_{fc}$ ) was calculated by:

$$N_{fc} = \frac{(V_t/V_a) \cdot (M_f \cdot N_f)}{(M_{start} \cdot N_{start})}$$

Where  $N_f$  = the nitrogen content in the fraction and  $N_{start}$  = the nitrogen content in the starting material. Protein yield was estimated by:

$$N_{fc} \cdot \frac{N_{tot}}{C_{w,start}} \cdot K_b$$

Where  $C_{w,start}$  = the dry matter content of the freeze-dried starting material and  $K_b$  stands for the empirically determined ratio between amino acids (AAs) and total biomass nitrogen ( $N_{tot}$ ), i.e. 4.9 for *Azolla* species [8], 4.6 for *U. lactuca* [23], 4.8 for *L. minor* [24] and 5.7 for Soybean [25].

Lastly protein purity was estimated by multiplying the nitrogen content ( $N_f$ ) with  $K_{AA}$ , which is the ratio between AAs and amino acid nitrogen ( $N_{AA}$ ) [8], i.e. 5.7 for *Azolla* species [8] and *U. lactuca* [23] and 5.8 for *L. minor* [24] and soybeans [25]. Both  $K_b$  and  $K_{AA}$  have not previously been determined for *S. molesta* and were assumed equal to the ratios for *Azolla*.

Quantification of pyrolysis and THM products was performed by peak integration of mass chromatograms using characteristic fragment ions of the compounds of interest. A list of retention times and characteristic fragment ions used for quantification is provided in **Table S1, Supporting information**.

### 3. Results & discussion

#### 3.1. Growth and chemical composition of *A. filiculoides*

*Azolla filiculoides* cultures grew under constant climatic conditions and from each culture 33% of the biomass, corresponding to roughly 20 g of fresh biomass was harvested weekly. Biomass productivity averaged 3.4 g dry weight  $m^{-2} day^{-1}$  over a period of 28 days. Crude protein content was estimated protein content at 20.6% of the dry weight (% for the remainder of the article). The biomass further contained 20.5% polysaccharides, predominantly glucan (14.0%) followed by galactan (2.5%), xylan (1.5%) and mannan (1.3%) (Table 1). Lipid extraction yielded 12.8% of crude lipids, whilst extraction with aqueous acetone (70 v/v%) yielded 3.4% tannic acid equivalent of (poly)phenols. Hot water extraction also yielded 2.1% of soluble sugars, mostly fructose and glucose. The biomass further contained 14.3% of ash, of which 11.9% was soluble by hot water extraction (Table 1).

The ash content is within the range (8.7–18.5%) previously reported for *A. filiculoides* [26,27]. The protein content determined here differs from values reported previously [7,26,27], since we used a conversion ratio ( $K_b$ ) specific for *Azolla* (4.9), instead of the standard 6.25 [8]. Hemicellulose content (10.8–18.1%) and cellulose content (11.0–22.7%) were previously determined by the Van Soest method [26], which can overestimate polysaccharide

**Table 1**  
Chemical composition of *A. filiculoides*.

Compounds	Content (% of dry weight)
Crude protein <sup>1</sup>	20.6
Lipids	12.8
Total phenol <sup>2</sup>	3.4
Soluble sugars	2.1
Fructose	1.20
Galactose	0.04
Glucose	0.70
Xylose	0.15
Structural carbohydrates	20.5
Glucan	14.0
Xylan	1.5
Galactan	2.5
Arabinan	0.7
Mannan	1.3
Rhamnan	0.6
Ash	14.3

<sup>1</sup> Based on a ratio between protein and total N of 4.9.

<sup>2</sup> In tannic acid equivalent.

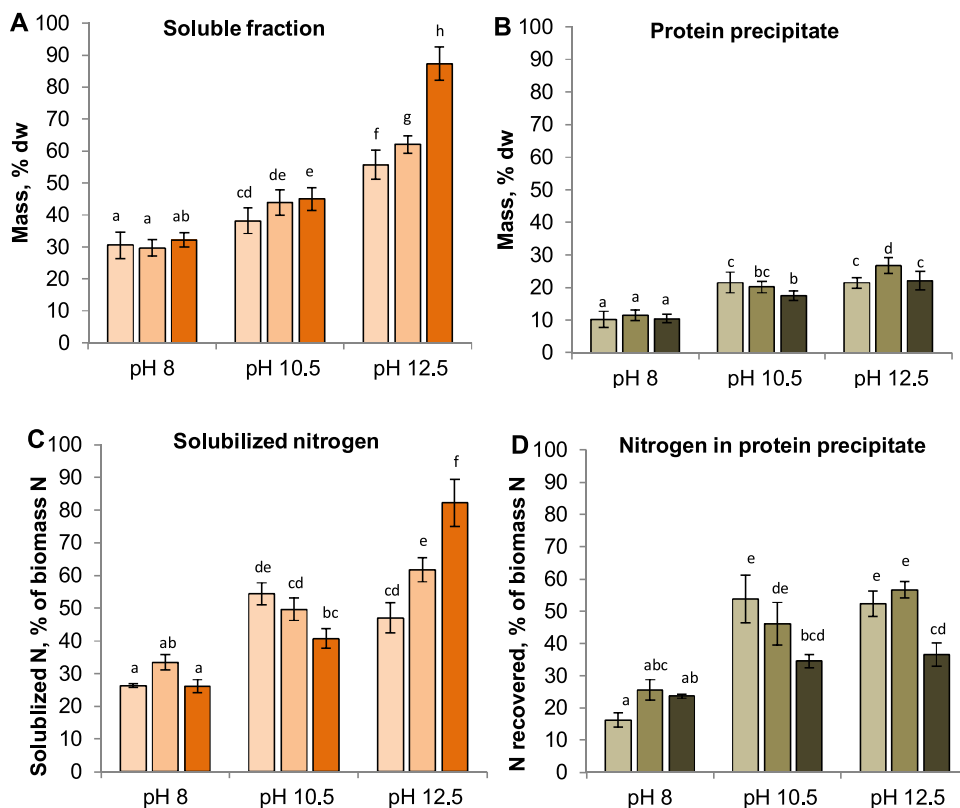
content due to protein remaining in the Neutral Detergent Fiber residue [28]. Our work indicates a lower content of polysaccharides in *A. filiculoides*, and in particular less hemicellulose than was previously estimated by the Van Soest method.

#### 3.2. Alkaline extraction at pH 10.5 effectively solubilizes *Azolla* protein

The harvested biomass was used to test a series of extractions at pH 8, 10.5 and 12.5 carried out either at RT, 45 °C or 95 °C. As illustrated in Fig. 1A,B, pH had a pronounced effect on the mass and nitrogen (N) balances of the extraction. At the mildest conditions of pH 8 and RT only 31% of the biomass dry weight was solubilized and 10% was precipitated. Of the biomass N, 31% was solubilized and 18% was recovered in the protein precipitate (Fig. 1C, D). Increasing the pH to 10.5 yielded more solubilized compounds, which were mostly recovered in the protein precipitate (23%). The increase in pH preferentially solubilized N-rich compounds and acid precipitation recovered 54% of the initial biomass N in the protein precipitate. Increasing the pH from pH 10.5 to 12.5 at RT increased solubilized compounds, but did not increase the mass of the protein precipitate (21%) (Fig. 1A,B, Figure S1, Supporting information). Moreover, both the amount of solubilized N and the amount of N recovered in the protein precipitate was similar at pH 12.5 and pH 10.5 (Fig. 1C,D).

The effect of temperature was specific for each extraction pH. At pH 8 a slight increase in temperature from RT to 45 °C seemingly increased (non-significant) the biomass N recovered in the protein precipitate, but no changes occurred when increasing temperature to 95 °C. At pH 10.5 elevated temperature had a slightly negative effect on both solubilized N and, especially, on the N recovered in the protein precipitate. For pH 12.5 extractions, temperature had a much greater effect. At a temperature of 45 °C the soluble fraction contained 62% of the initial mass and 67% of the biomass N. The protein precipitate represented 27% of the initial mass and contained 57% of the biomass N. At 95 °C the soluble fraction increased further to 82%, containing 84% of the biomass N. The solubilized compounds, however, were not preferably precipitated with acid but instead remained in the supernatant, resulting in a reduction in mass and N recovered in the protein precipitate when compared to extraction at 45 °C. (Fig. 1, Figure S1, Supporting Information).

Extracting at pH 12.5 and 95 °C was previously reported to provide high protein yields from green tea leaves [29]. The improved solubilization of N is likely due to the degradation of membrane-bound protein that would otherwise not become



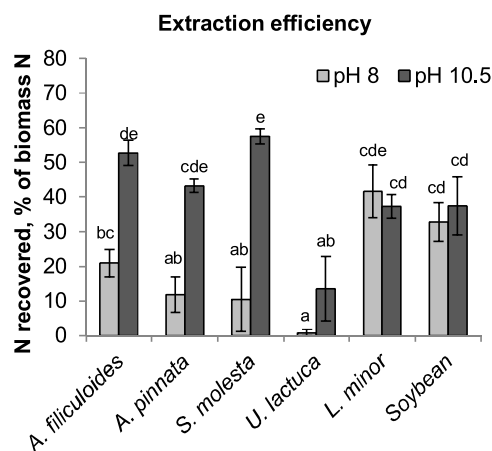
**Fig. 1.** Percentage of initial mass ( $n=9$ ) and nitrogen ( $n=3$ ) in the soluble fraction (A,C) and protein precipitate (B,D) obtained by alkaline extraction of *A. filiculoides*. Extractions were performed at pH 8, pH 10.5 and 12.5 for 4 h. Lighter to darker bars indicate extraction temperature: RT, 45 °C and 95 °C. Statistical analysis was by one-way ANOVA and a tukey post-hoc test; letters indicate significantly differing groups ( $P < 0.05$ ).

soluble. Considerable degradation of *Azolla* protein was clearly observed after 2 h of extraction at pH 12.5 and 95 °C (Figure S2, Supporting Information). However, the degraded protein may no longer be susceptible to acid precipitation, explaining the small (41%) fraction of solubilized N being precipitated. Hence extraction at pH 12.5 and 95 °C does not improve protein yield compared to extraction performed at pH 10.5 and RT, at least not for the extraction time tested and when used in combination with acid precipitation.

### 3.3. Comparing alkaline extraction of protein from *Azolla* biomass and other (aquatic) feedstocks

A second set of protein extractions was performed to compare protein yields from *Azolla* to other aquatic weeds and macroalgae. The crude protein content of the aquatic weeds and macroalgae ranged between 16% (*A. pinnata*) to 25% (*L. minor*), with *A. filiculoides* containing 21%, whilst soybeans had a much higher content of 37% (Table S1, Supporting Information). The extraction efficiency from *U. lactuca* was very low compared to other feedstocks: at pH 8 almost no protein pellet was obtained, whereas at pH 10.5 only 7% of the mass and 13.5% of the nitrogen ended up in the protein pellet (Fig. 2, Table S1, Supporting Information). The difference when compared to other feedstocks is likely due to the tough cell wall of algae, which is generally difficult to disrupt [30,31].

For the aquatic ferns *A. filiculoides*, *A. pinnata* and *S. molesta*, extraction efficiency was low at pH 8, but increased significantly when an extraction pH of 10.5 was used, i.e. 2.5, 3.7 and 5.5 fold respectively. Recovery of N from *A. filiculoides* at pH 10.5 was similar to the previous experiment, recovering 53% of the biomass N in the protein precipitate (Fig. 2).



**Fig. 2.** The effect of extraction pH on the amount of nitrogen recovered from protein extractions at RT of biomass from *A. filiculoides*, *A. pinnata*, *S. molesta*, *U. lactuca*, *L. minor*, and soybean (*Glycine max* seeds) ( $n=3$ ). Statistical analysis was by one-way ANOVA and a tukey post-hoc test; letters indicate significantly differing groups ( $P < 0.05$ ).

For the duckweed *L. minor* at pH 8 already 42% of the nitrogen was recovered in the protein precipitate. Unlike the other aquatic plants, increasing the extraction pH to 10.5 did not significantly change the extraction efficiency (Fig. 2). Similarly, for soybeans no significant change was observed between an extraction pH of 8 and 10.5. At pH 8 an extraction efficiency of 37% was obtained. However, the mechanical destruction of the soybeans proved more difficult compared to the leaf tissues and consequently this extraction efficiency is at the low end of values reported in literature (33–71%) [30].



Given the aquatic plants tested here consist mostly of leaf tissue, the maximum extraction efficiencies may correspond to the water soluble protein fraction, generally found to lie between 40–50% of biomass N in leaves, and of which the RuBisCo protein generally represents half [32–34]. Membrane-associated proteins such as thylakoid components, representing the largest part of the insoluble fraction of leaves [35] are unlikely solubilized during extraction at pH 8 and pH 10.5 at RT.

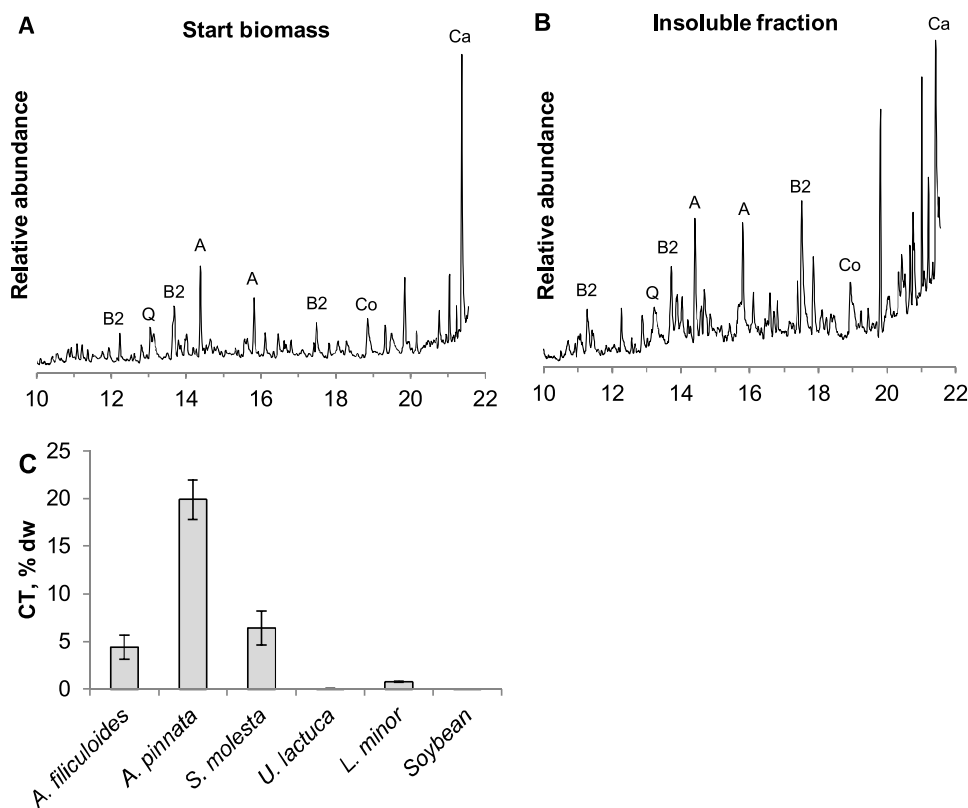
Absolute protein yields were highest for *A. filiculoides* (13.3%) at pH 10.5, followed by soybeans at pH 8 (13.0%), *S. molesta* at pH 10.5 (12.4%) and *L. minor* at pH 8 (12.2%). Maximum protein yield from *A. pinnata* was significantly less (8.1%) compared to *A. filiculoides*, likely due to the lower protein content in the biomass (Table S1, Supporting information). The purity of the protein pellet from pH 8 extractions of *L. minor* (68%) and soybean (65%), however, was estimated to be much higher than that to the pH 10.5 extractions from the aquatic ferns *A. filiculoides* (52%), *A. pinnata* (29%) and *S. molesta* (46%) (Table S1, Supporting information). Mechanical extraction of *Azolla* biomass followed by heat coagulation was previously tested on aquatic weeds, but absolute protein yields were not reported [16,36]. The nitrogen content of extracts obtained using the latter method were 11.4% for *A. filiculoides*, 6.3% for *A. pinnata* and 6.1% for *L. minor*, compared to 9.1%, 5.1% and 11%, respectively, obtained by pH 10.5 extraction performed in this study [16,36].

Hence at pH 8 alkaline protein extraction results in a low extraction efficiency in all aquatic ferns of the *Salviniaceae* family, but a high efficiency in duckweed *L. minor* and soybeans. Elevating the extraction pH to 10.5 allows to obtain equal or higher extraction efficiencies from the aquatic ferns. The protein purity in the extracts obtained at pH 10.5 is lower in *Azolla* and *Salvinia*

compared to *L. minor* and soybean, and slightly lower than what has been previously reported for *Azolla* using an alternative extraction method. Further research would be needed to evaluate the overall performance of alkaline extraction compared to other protein extraction methods for aquatic weeds.

### 3.4. Lower extraction yield at pH 8 in *Azolla* is linked to condensed tannins

It was hypothesized that the high polyphenol content of *Azolla* could be the cause for the low extraction efficiency at pH 8 compared to duckweed and soybeans. To determine the type of the (poly)phenols that remain insoluble during extraction alongside the proteins, the insoluble fractions obtained from pH 8 extraction at RT were investigated by Thermally assisted Hydrolysis and Methylation (THM). In both the starting biomass and the insoluble fraction, breakdown products were identified of the flavonoid A ring (1,3,5-trimethoxybenzene, 1-methyl-2,4,6-trimethoxybenzene and 1-ethyl-2,4,6-trimethoxybenzene), and of a flavonoid B-ring with two hydroxyl groups (B2) (1,2-dimethoxybenzene, 4-methyl-1,2-dimethoxybenzene, 1,2,4-trimethoxybenzene, 3,4-dimethoxybenzaldehyde, 3,4-dimethoxybenzoic acid methyl ester), which are typical of the catechin-type condensed tannins (CTs) identified in *Azolla* previously [37] (Fig. 3A,B). Compared to the other THM products from phenolic compounds, such as methylated caffeic acid (Ca) and coumaric acid (Co) and 1,4-dimethoxybenzene from quinic acid (Q), the breakdown products from the A and B ring of CTs were enriched in the insoluble fraction after protein extraction at pH 8 (Table S2, Supporting Information). CTs were therefore particularly abundant in the insoluble fraction.



**Fig. 3.** Presence of condensed tannins (CTs) in *Azolla* biomass and other feedstocks. (A) THM chromatograms showing THM products of phenolic compounds in the starting biomass and (B) the insoluble fraction after extraction at pH 8. Labels indicate the likely origin, i.e. A = flavonoid A-ring, B2 = flavonoid B-ring with two hydroxyl groups, Ca = Caffeic acid, Co = Coumaric acid and Q = Quinic acid, specific compounds and retention times are given in Table S2, Supporting Information. (C) CT content of *A. filiculoides*, *A. pinnata*, *S. molesta*, *U. lactuca*, *L. minor* and soybeans, as determined by the acid butanol assay.

To evaluate whether the CT content of the biomass can explain the decreased extractability of protein at pH 8, the concentration of CTs were determined in the batches of biomass of *Azolla* and other feedstocks which were used for protein extraction experiments. The aquatic ferns all contained CTs, with *A. filiculoides* and *S. molesta* containing 4.4% and 6.4% respectively, whereas *A. pinnata* contained a staggering 19.9% CTs. In contrast, no CTs were measured in soybeans and *U. lactuca* and only 0.8% in the duckweed *L. minor* (Fig. 3C). Hence, with the exception of the macroalgae *U. lactuca*, the higher content of CTs in the biomass seems to correlate with low extraction efficiency at pH 8 versus pH 10.5.

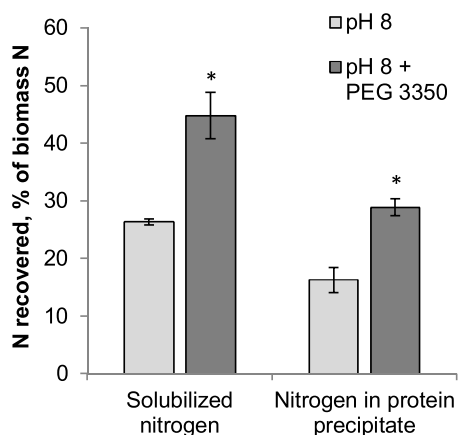
### 3.5. Elevating pH may enhance extraction yield by preventing protein precipitation by CTs

CTs are well known to precipitate protein, depending on pH, temperature, protein sequence, and functional groups and the degree of polymerization of the CTs [38,39]. The model protein Bovine Serum Albumine (BSA) was mostly used to study protein and CT interactions, but Zeller et al. (2015) showed that CTs precipitate Alfalfa leaf protein more strongly than BSA [39]. CTs also strongly precipitated purified Rubisco [40,41]. Interestingly, increasing pH was found to decrease the precipitation of protein by CTs [42]. Specific compounds, such as polyethylene glycol (PEG), can also prevent the interaction between tannins and protein [41]. Hence to test whether the increase in pH could improve protein extraction efficiency by means of decreasing the precipitation of protein by CTs, we performed alkaline protein extraction (pH 8) of *Azolla* without and with 1% PEG 3350 (Fig. 4). The addition of PEG during protein extraction resulted in a significant increase in solubilized N and N recovered in the protein precipitate (Fig. 4).

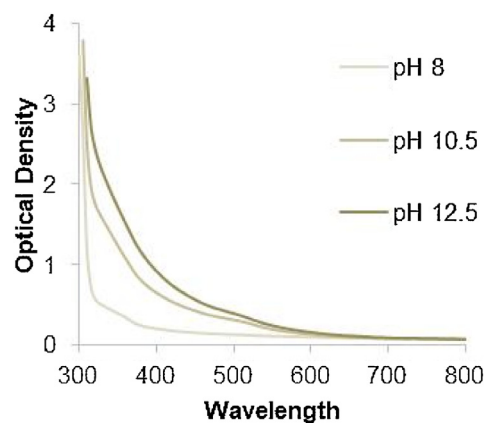
Hence, although it is known that increasing pH can lead to higher protein extraction efficiency by enhancing protein solubility and aiding in cell wall lysis [30], our data suggests that in *Azolla* increasing pH may enhance protein extraction yield by preventing protein precipitation by CTs.

### 3.6. Elevating pH causes polyphenol oxidation in the protein extract

Although increasing the pH elevates protein yield from *Azolla* we observed that extraction at pH 10.5 or 12.5, unlike pH 8, resulted in fast red-brown coloration of the protein extract (Fig. 5).



**Fig. 4.** The effect of the addition of PEG on nitrogen solubilization and recovery at pH 8. Nitrogen content of the soluble fraction and protein precipitate were determined for pH 8 extractions at RT with and without 1% w/v PEG (MW 3350) (n = 3). The asterix denotes a significant (p < 0.05) difference as determined by an independent sample T-test.



**Fig. 5.** Color spectra of the protein extracts. Coloration of the protein extract was determined by photospectrometer in diluted (1:10) protein extracts obtained by pH 8, pH 10.5 and pH 12.5 extractions at RT.

This bathochromic shift in the UV/VIS absorption spectra to the visible spectrum is typical of oxidative polymerization of (poly) phenols; in addition the rate of oxidation increases exponentially with pH [43] (Figure S3; Figure S4, Supporting Information). Oxidation of (poly)phenols proceeds by the formation of highly reactive quinones [44,45]. When in the vicinity of proteins, quinones irreversibly react with the sulfhydryl and amino groups of proteins and thereby form protein cross-links [44,46,47]. Additionally, quinones can undergo condensation reactions, resulting in the formation of high molecular weight pigments [45].

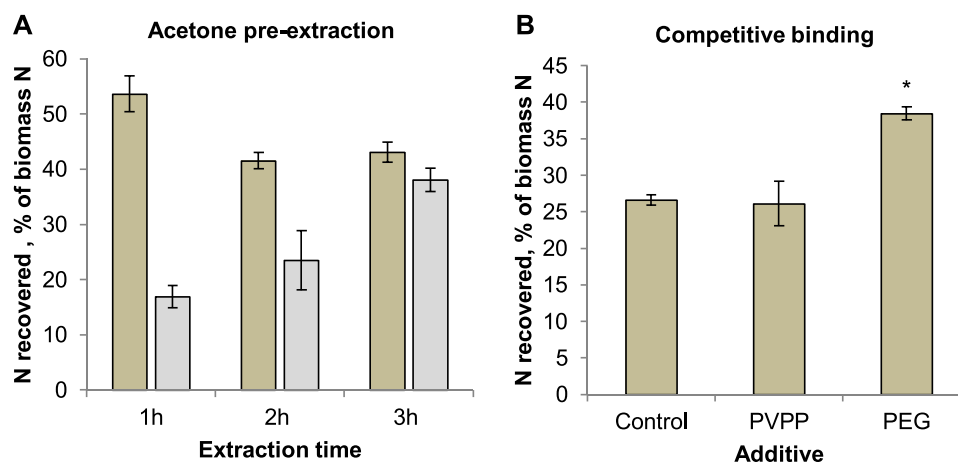
The coloration of the protein precipitate thus suggests that considerable amounts of polyphenols are present in the protein pellet and have likely formed covalent bonds with protein, which would obstruct any attempts of subsequent separation [48]. Indeed, various attempts were made to separate tannins and protein from the protein pellet, as well as reference mixtures of BSA and spruce condensed tannins, at high pH, but without success, even when a strong antioxidant (ascorbate) was added to prevent polyphenol oxidation (Table S3, Supporting information).

Hence, extraction of protein from *Azolla* at elevated pH seems to inevitably yield a protein product containing a high degree of (oxidized) polyphenols, including condensed tannins. This may lead to a lower quality product compared to other feedstocks for which lower pH extraction can be employed. For example the quinone–amino group reactions are known to decrease the digestibility and bioavailability of protein-bound lysine and cysteine, the latter was found one of the first limiting amino acids when using *Azolla* as feed [8,46].

### 3.7. Alternative approaches to extract protein from *Azolla*

As separation of polyphenols and protein from alkaline solution proved unsuccessful, we evaluated two alternative approaches to enhance protein extraction yield, while also reducing the binding of protein by CTs. The first approach included the pre-extraction of CTs followed by protein extraction. The second approach explored protein extraction in the presence CT binding additives. To improve the relevance of the data produced in terms of scalability, we increased the scale of the protein extractions to 2 g dw per extraction and performed extractions directly on wet biomass in a laboratory blender.

Pre-extraction of (poly)phenolic compounds by aqueous acetone (70%) decreased the content of CTs by 76–85% (Figure S5, Supporting Information). However, the pre-extraction with acetone denatured the protein, making them insoluble at pH



**Fig. 6.** Protein yield and removal of (poly)phenols by aqueous acetone pre-extraction (A) and competitive binding (B). (A) The amount of biomass N recovered in the protein precipitate obtained by extraction at pH 12.5 and 95 °C, without (brown) and with (gray) acetone pre-extraction (n = 3). (B) Nitrogen recovery after extractions at pH 8 and 45 °C performed with no additive (control), 1.5% w/v PVPPP and 1.5% w/v PEG (n = 3). The asterisk denotes a significant ( $p < 0.05$ ) difference to the control as determined by an independent sample *t*-test.

10.5. A subsequent protein extraction at pH 12.5 and 95 °C did allow solubilization of protein over time, recovering 38% of biomass N in the protein precipitate after 3 h (Fig. 6A). The partial degradation of protein at pH 12.5 and 95 °C is utilized to bring denatured protein back in solution. In contrast, without aqueous acetone pre-extraction, proteins are directly soluble and a maximum of 54% of the biomass N is recovered after 1 h, whereas further exposure to pH 12.5 and 95 °C negatively affected recovery.

The harsh conditions needed to extract protein after aqueous acetone pre-extraction may evoke various chemical reactions between protein and other solubilized compounds that negatively influence protein quality. The coloration of the protein precipitate indicated that the (poly)phenols that remained in the biomass after aqueous acetone pre-extraction were oxidized. Furthermore, harsh alkali treatment was shown to promote formation of non-utilizable amino acids and oxidation, which can reduce the bioavailability of cysteine [30,46].

A better quality protein extract may be obtained when using a lower extraction pH in combination with a CT binding additive. Similar to the small scale extraction described earlier (Fig. 4) we added PEG while extracting protein at pH 8. To enhance protein solubility we performed the extraction at 45 °C and used 1.5% w/v PEG with a molecular weight of 6000 Da, which was reported to bind CTs better than the smaller PEGs [49]. Polyvinylpyrrolidone (PVPPP) is also known to bind CTs, although slightly less efficiently than PEG [49]. PVPPP was tested in its insoluble form, because this would permit easier recovery and recycling of PVPPP-CT complexes.

The extraction with PEG yielded a protein precipitate containing 38% of the initial N, which was significantly higher than the control for which 26% of the biomass N was recovered in the protein precipitate (Fig. 6B). In contrast, extraction with 1.5% w/v insoluble PVPPP gave near identical results to the control and therefore did not improve protein solubilization at pH 8.

PEG's exceptionally strong binding affinity towards CTs has been attributed to the presence of weak hydrogen bond acceptors in the form of ether bonds combined with a hydrophilic ethyl chain [50]. Additionally the polymeric nature of PEG allows it to connect to the polymeric tannins at multiple sites. PVPPP was previously shown to bind CTs more strongly than protein [42]. Unlike PEG, the applied PVPPP was insoluble, which may (partly) explain the lack of improvement in protein yield as it may not come into contact with CTs quickly enough after cell lysis, allowing the released CTs time to bind to protein first.

Feeding animals PEG next to a tannin-rich diet, has been shown to alleviate the negative effects of tannins-protein interactions in the animals gut [51,52]. Analogously, binding of CTs by PEG during protein extraction may already enhance the digestibility of the protein extracts, which would permitting their use as high-quality feed [51,52]. The costs of adding 1–1.5% PEG to the extraction process, however, would likely prohibit the practice. Instead more work on the recovery of PEG is likely required to make the process economically feasible.

### 3.8. Co-products from an *Azolla* protein extraction

Multiple approaches may be taken to extract protein from the tannin-rich *Azolla* biomass and these will differ in processing costs and product quality. The choice of processing methods, however, will also depend on the added value of the co-products. A major co-product is the insoluble fraction which is rich in carbohydrates: analysis of a hydrolysate of insoluble fraction obtained after pH 10.5 protein extractions, contained 31.7% monosaccharides (Table S4, Supporting Information). This hydrolysate may be very useful for conversion into bio-ethanol, since *Azolla* contains only trace amounts, if any, of true lignin, which is favorable since lignin removal via pre-treatment is a major cost for producing ethanol from lignocellulosic biomass [37,53,54].

Additionally, extraction methods that separate CTs from protein may be combined with downstream purification steps to yield purified CTs. Such a process could be used to replace part of the 180,000 t of CTs produced annually, mostly by hot-water extraction of tree barks [55], dominantly used in the leather industry. Demand for CTs may further increase in the future as novel applications are developed, such as using CTs as replacement of fossil-based phenol in insulating foams and adhesives [55,56].

## 4. Conclusions

Alkaline protein extraction from *Azolla* biomass at mild pH seems largely limited by high concentration of condensed tannins (CTs). Increasing the extraction pH to 10.5 was shown to enhance protein yields, which may be due to reduced protein-CT precipitation. However, subsequent separation of CTs and protein were unsuccessful. Two methods were introduced to enhance protein yields while reducing the binding of protein by CTs: (1) acetone pre-extraction followed by alkaline protein extraction at pH 12.5 and 95 °C and (2) extraction at pH 8 in the presence of CT-

binding polyethylene glycol (PEG). With both methods 38% of the biomass nitrogen could be recovered in the protein precipitate. Whilst acetone pre-extraction is effective in removing CTs, it requires harsh extraction conditions to re-solubilize protein. Addition of PEG reduces CT-protein precipitation under mild conditions, yet recovery of the PEG will be needed to make the process economically feasible. To conclude alkaline protein extraction can be used to extract protein from *Azolla*, but requires additional processing compared to other feedstocks, such as duckweed and soybeans. CTs may, however, also prove to be a valuable co-product of alkaline extraction of *Azolla* and similar tannin-rich feedstocks.

### Declaration of Competing Interest

None.

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### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2019.e00368>.

### References

- [1] United Nations World Population Prospects: The 2015 Revision, Key Findings and Advance Tables, Working Paper No. ESA/P/WP.241, 2015.
- [2] D.J. Millward, A.A. Jackson, Protein/energy ratios of current diets in developed and developing countries compared with a safe protein/energy ratio: implications for recommended protein and amino acid intakes, *Public Health Nutr.* 7 (2004) 387–405.
- [3] WWF, The Growth of Soy: Impacts and Solutions, WWF International, Gland, Switzerland, 2014.
- [4] T.A. Lumpkin, D.L. Plucknett, *Azolla*: botany, physiology, and use as a green manure, *Econ. Bot.* 34 (1980) 111–153.
- [5] C. Van Hove, A. Lejeune, The *Azolla*-anabaena symbiosis, *Biol. Environ.* 102 (2002) 23–26.
- [6] C. van Hove, *Azolla*: and Its Multiple Uses With Emphasis on Africa, FAO, Rome, Italy, 1989.
- [7] M. Becerra, E. Murgueitio, G. Reyes, T.R. Preston, *Azolla filiculoides* as partial replacement for traditional protein supplements in diets for growing fattening pigs based on sugar cane juice, *Livestock Research for Rural Development* 2 (1990) 15–22.
- [8] P. Brouwer, H. Schluempmann, K.G.J. Nierop, J. Elderson, P.K. Bijl, I. van der Meer, W. de Visser, G.-J. Reichart, S. Smeekens, A. van der Werf, Growing *Azolla* to produce sustainable protein feed: the effect of differing species and CO<sub>2</sub> concentrations on biomass productivity and chemical composition, *J. Sci. Food Agric.* 98 (12) (2018) 4759–4768.
- [9] P. Leterme, A.M. Londoño, D.C. Ordoñez, A. Rosales, F. Estrada, J. Bindelle, A. Buldgen, Nutritional value and intake of aquatic ferns (*Azolla filiculoides* Lam. And *Salvinia molesta* Mitchell.) in sows, *Anim. Feed Sci. Technol.* 155 (2010) 55–64.
- [10] B. Basak, A.H. Pramanik, M.S. Rahman, S.U. Tarafdar, B.C. Roy, *Azolla* (*Azolla pinnata*) as a feed ingredient in broiler ration, *Int. J. Poult. Sci.* 1 (2002) 29–34.
- [11] H. Naghshi, S. Khojasteh, M. Jafari, Investigation of the effect of different levels of *Azolla* (*Azolla pinnata*) on performance and characteristics of Cobb Broiler Chicks, *International Journal of Farming and allied sciences* 3 (2014) 45–49.
- [12] O.A. Alalade, E.A. Iyayi, Chemical composition and the feeding value of *Azolla* (*Azolla pinnata*) meal for egg-type chicks, *Int. J. Poult. Sci.* 5 (2006) 137–141.
- [13] E. Sjöding, The *Azolla* Cooking and Cultivation Project, Erik Sjöding, 2012.
- [14] Y.W. Sari, W.J. Mulder, J.P.M. Sanders, M.E. Bruins, Towards plant protein refinery: review on protein extraction using alkali and potential enzymatic assistance, *Biotechnol. J.* 10 (2015) 1138–1157.
- [15] L.L. Rusoff, E.W. Blakeney Jr., D.D. Culley Jr., Duckweeds (*Lemnaceae* family): a potential source of protein and amino acids, *J. Agric. Food Chem.* 28 (1980) 848–850.
- [16] E.A. Fasakin, Nutrient quality of leaf protein concentrates produced from water fern (*Azolla africana* Desv.) and duckweed (*Spirodela polyrrhiza* L. Schleiden), *Bioresour. Technol.* 69 (2) (1999) 185–187.
- [17] K. Lorenz, C.M. Preston, Characterization of high-tannin fractions from humus by carbon-13 cross-polarization and magic-angle spinning nuclear magnetic resonance, *J. Environ. Qual.* 31 (2002) 431–436.
- [18] K.G.J. Nierop, C.M. Preston, J. Kaal, Thermally assisted hydrolysis and methylation of purified tannins from plants, *Anal. Chem.* 77 (2005) 5604–5614.
- [19] P.G. Waterman, S. Mole, *Analysis of Phenolic Plant Metabolites*, Blackwell Scientific Publications, Oxford, 1994.
- [20] H.P.S. Makkar, G. Gamble, K. Becker, Limitation of the butanol-hydrochloric acid-iron assay for bound condensed tannins, *Food Chem.* 66 (1999) 129–133.
- [21] R.J.H. Grisel, J.C. Van Der Waal, E. De Jong, W.J.J. Huijgen, Acid catalysed alcoholysis of wheat straw: towards second generation furan-derivatives, *Catal. Today* 223 (2014) 3–10.
- [22] J. Wildschut, A.T. Smit, J.H. Reith, W.J.J. Huijgen, Ethanol-based organosolv fractionation of wheat straw for the production of lignin and enzymatically digestible cellulose, *Bioresour. Technol.* 135 (2013) 58–66.
- [23] P. Bikker, M.M. van Krimpen, P. van Wikselaar, B. Houweling-Tan, N. Scaccia, J. W. van Hal, W.J.J. Huijgen, J.W. Cone, A.M. López-Contreras, Biorefinery of the green seaweed *Ulva lactuca* to produce animal feed, chemicals and biofuels, *J. Appl. Phycol.* (2016) 1–15.
- [24] Y. Zhao, Y. Fang, Y. Jin, J. Huang, S. Bao, T. Fu, Z. He, F. Wang, H. Zhao, Potential of duckweed in the conversion of wastewater nutrients to valuable biomass: a pilot-scale comparison with water hyacinth, *Bioresour. Technol.* 163 (2014) 82–91.
- [25] K.J. Petzke, I.E. Ezeagu, J. Proll, A.O. Akinsoyinu, C.C. Metges, Amino acid composition, available lysine content and in vitro protein digestibility of selected tropical crop seeds, *Plant Foods Hum. Nutr.* 50 (1997) 151–162.
- [26] M.L. Costa, M.C. Santos, F. Carrapiço, Biomass characterization of *Azolla filiculoides* grown in natural ecosystems and wastewater, *Hydrobiologia* 415 (1999) 323–327.
- [27] S.N. Datta, Culture of *Azolla* and its efficacy in diet of *Labeo rohita*, *Aquaculture* 310 (2011) 376–379.
- [28] B. Godin, R. Agneessens, P. Gerin, J. Delcarte, Structural carbohydrates in a plant biomass: correlations between the detergent fiber and dietary fiber methods, *J. Agric. Food Chem.* 62 (2014) 5609–5616.
- [29] C. Zhang, J.P.M. Sanders, M.E. Bruins, Critical parameters in cost-effective alkaline extraction for high protein yield from leaves, *Biomass Bioenergy* 67 (2014) 466–472.
- [30] Y.W. Sari, W.J. Mulder, J.P.M. Sanders, M.E. Bruins, Towards plant protein refinery: review on protein extraction using alkali and potential enzymatic assistance, *Biotechnol. J.* 10 (2015) 1138–1157.
- [31] M. Polikovskiy, F. Fernand, M. Sack, W. Frey, G. Müller, A. Golberg, Towards marine biofineries: selective protein extractions from marine macroalgae *Ulva* with pulsed electric fields, *Innov. Food Sci. Emerg. Technol.* 37 (2016) 194–200.
- [32] T. Takashima, K. Hikosaka, T. Hirose, Photosynthesis or persistence: nitrogen allocation in leaves of evergreen and deciduous *Quercus* species, *Plant Cell Environ.* 27 (2004) 1047–1054.
- [33] J.L. Funk, L.A. Glenwinkel, L. Sack, Differential allocation to photosynthetic and non-photosynthetic nitrogen fractions among native and invasive species, *PLoS One* 8 (2013).
- [34] R. Fiorentini, C. Galoppini, The proteins from leaves, *Plant Foods Hum. Nutr.* 32 (1983) 335–350.
- [35] J.R. Evans, J.R. Seeman, The allocation of protein nitrogen in the photosynthetic apparatus: costs, consequences and control, in: W.R. Briggs (Ed.), *Photosynthesis*, Alan R. Liss, New York, 1989 pp. 183–184–205.
- [36] A. Dewanji, Amino Acid Composition of Leaf Proteins Extracted from Some Aquatic Weeds, *J. Agric. Food Chem.* 41 (1993) 1232–1236.
- [37] K.G.J. Nierop, E.N. Speelman, J.W. de Leeuw, G.-J. Reichart, The omnipresent water fern *Azolla caroliniana* does not contain lignin, *Org. Geochem.* 42 (2011) 846–850.
- [38] M. Saminathan, H.Y. Tan, C.C. Sieo, N. Abdullah, C.M.V.L. Wong, E. Abdulmalek, Y.W. Ho, Polymerization degrees, molecular weights and protein-binding affinities of condensed tannin fractions from a leucaena leucocephala hybrid, *Molecules* 19 (2014) 7990–8010.
- [39] W.E. Zeller, M.L. Sullivan, I. Mueller-Harvey, J.H. Grabber, A. Ramsay, C. Drake, R.H. Brown, Protein precipitation behavior of condensed tannins from *Lotus pedunculatus* and *Trifolium repens* with different mean degrees of polymerization, *J. Agric. Food Chem.* 63 (2015) 1160–1168.
- [40] T.A. McAllister, T. Martinez, D.B. Hee, A.D. Muir, L.J. Yanke, G.A. Jones, Characterization of condensed tannins purified from legume forages: chromophore production, protein precipitation, and inhibitory effects on cellulose digestion, *J. Chem. Ecol.* 31 (2005) 2049–2068.
- [41] W.C. McNabb, J.S. Peters, L.Y. Foo, G.C. Waghorn, F.S. Jackson, Effect of condensed tannins prepared from several forages on the in vitro precipitation of ribulose-1,5-bisphosphate carboxylase (Rubisco) protein and its digestion by trypsin (EC 2.4.21.4) and chymotrypsin (EC 2.4.21.1), *J. Sci. Food Agric.* 77 (1998) 201–212.
- [42] A.E. Hagerman, L.G. Butler, The specificity of proanthocyanidin-protein interactions, *J. Biol. Chem.* 256 (1981) 4494–4497.
- [43] P.K. Jha, G.P. Halada, The catalytic role of uranyl in formation of polycatechol complexes, *Chem. Cent. J.* 5 (1) (2011).



- [44] C. Le Bourvellec, C.M.G.C. Renard, Interactions between polyphenols and macromolecules: quantification methods and mechanisms, *Crit. Rev. Food Sci. Nutr.* 52 (2012) 213–248.
- [45] T. Ozdal, E. Capanoglu, F. Altay, A review on protein-phenolic interactions and associated changes, *Food Res. Int.* 51 (2013) 954–970.
- [46] S. Damodaran, Amino acids, peptides, and proteins, in: O.R. Fennema (Ed.), *Food Chemistry*, Marcel Dekker, Inc., 1996, pp. 321–429.
- [47] T. Prodpran, S. Benjakul, S. Phatcharat, Effect of phenolic compounds on protein cross-linking and properties of film from fish myofibrillar protein, *Int. J. Biol. Macromol.* 51 (2012) 774–782.
- [48] A. Khoddami, M.A. Wilkes, T.H. Roberts, Techniques for analysis of plant phenolic compounds, *Molecules* 18 (2013) 2328–2375.
- [49] H.P.S. Makkar, M. Blümmel, K. Becker, Formation of complexes between polyvinyl pyrrolidones or polyethylene glycols and tannins, and their implication in gas production and true digestibility in *in vitro* techniques, *Br. J. Nutr.* 73 (1995) 897–913.
- [50] H.I. Oh, J.E. Hoff, G.S. Armstrong, L.A. Haff, Hydrophobic interaction in tannin-protein complexes, *J. Agric. Food Chem.* 28 (1980) 394–398.
- [51] K. Yisehak, K. Biruk, B. Abegaze, G.P.J. Janssens, Growth of sheep fed tannin-rich *Albizia gummifera* with or without polyethylene glycol, *Trop. Anim. Health Prod.* 46 (2014) 1113–1118.
- [52] R. Bhatta, A.K. Shinde, S. Vaithyanathan, S.K. Sankhyan, D.L. Verma, Effect of polyethylene glycol-6000 on nutrient intake, digestion and growth of kids browsing *Prosopis cineraria*, *Anim. Feed Sci. Technol.* 101 (2002) 45–54.
- [53] C. Fu, J.R. Mielenz, X. Xiao, Y. Ge, C.Y. Hamilton, M. Rodriguez Jr., F. Chen, M. Foston, A. Ragauskas, J. Bouton, R.A. Dixon, Z.- Wang, Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass, *Proc. Natl. Acad. Sci. U. S. A.* 108 (2011) 3803–3808.
- [54] B. Yang, C.E. Wyman, Potential for low cost pretreatment of cellulosic biomass for biological production of fuels and chemicals, *TAPPI Int. Bioenergy Bioproducts Conf.* (2008).
- [55] K. Kemppainen, M. Siika-aho, S. Pattathil, S. Giovando, K. Kruus, Spruce bark as an industrial source of condensed tannins and non-cellulosic sugars, *Ind. Crops Prod.* 52 (2014) 158–168.
- [56] A. Pizzi, Recent developments in eco-efficient bio-based adhesives for wood bonding: opportunities and issues, *J. Adhes. Sci. Technol.* 20 (2006) 829–846.