Proteins from plant-based biomass: effects of post-harvest conditions on protein retention and quality

Part I

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Institute: Wageningen Food & Biobased Research

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Preface

This report describes the results of experiments within the project High value proteins from plant based biomass: effects of harvest, storage and transport on protein yield and quality (DFI-AF-19005), funded by the Ministry of Agriculture, Nature and Food Quality.

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On behalf of the project team,
Esther Hogeveen- van Echtelt
Project leader

Summary

This report represents an exploration into the potential impact of post-harvest technology to protein supply chains by studying the effects of post-harvest conditions on protein retention and quality in various types of crops. The research was performed independently by researchers from Wageningen Food & Biobased Research, funded by the Ministry of Agriculture, Nature and Food Safety by DFI- R&D budget, within the strategic WUR-KB theme of Healthy and Safe Food.

Development of robust supply chains of plant based proteins in Europe, requires the use of new protein crops, side streams of agricultural food crops, or non-food crops (water side plants, grass). A current blind spot within these supply chains, is the possible impact of post-harvest conditions on the protein yield, quality and functionality of fractions or isolated proteins.

In literature we found several indications that the timing of harvest, handling, storage and transport conditions of the raw material affect the final yield and quality of the different proteins (e.g. RuBisCo) present in the products. After harvest, loss of protein (quality) can occur due to proteolytic breakdown, denaturation of protein and through oxidative processes. These processes are complex and generally influenced largely by, amongst others, temperature and time. Yet, no systematic investigations into the effect of these factors during post-harvest stage before processing on protein content and quality have been done.

In experiments in this study we have looked to the effect of temperature and time after storage on protein retention and on Rubisco levels in Italian ryegrass and sugar beet leaves. Furthermore we have studied the effect of raw material short-term desiccation after harvest on protein retention and quality, using sugar beet leaves as key model crop.

In a storage experiment, Italian ryegrass (*Lolium multiflorum)* was stored at 20, 10, 5, 1, -1°C and 80% RH and sampled at various time intervals (0, 1, 3, 7, 14, 22 days). SDS-Page gels of extracts of these samples showed that proteins (specifically looking at the large subunit of Rubisco) are broken down faster at higher temperatures. At 10°C and more clearly at 20°C, most protein is broken down already within 7 days. At day 3, it seems that under all conditions (except for storage at 20°C), proteins are still intact. So low temperatures (between -1 to 5°C) can keep protein content and quality in grass stable for several days to weeks. The protein degradation was also reflected in a measured change of colour (ΔE) of the material.

In a following experiment, sugar beet leaves were stored at -5°C, 1°C, 10°C and 20°C and sampled after 0, 1, 3, 7, 14 and 21 days. Concerning the protein content, as measured with both BCA method and on protein gel, the storage temperature and duration seemed to have a limited effect within the first week, but showed a decrease in protein content at room temperature after 2 and 3 weeks of storage at 20ºC.
It seemed that the protein structure was affected by the storage conditions as the two rubisco subunits were not visible on the gel after 3 weeks of leaf storage at room temperature. The protein in the sugar beet leaves seemed to be stable for a period up to 2 weeks, independent of the storage temperature. From the present study, we can conclude that the protein remains stable for a relatively long period, especially when stored at lower temperatures, when sugar beet leaves are stored intact.

The samples from the sugar beet experiment have also been checked on changes in polyphenol content upon storage. It is known that polyphenols may form complexes with proteins leading to changes in the structural, functional and nutritional properties of both components (Ozdal et al., 2013). Measurement of total phenolic component in the samples (per gFW) indicated that there is an increase in levels starting from 7 days storage at 10ºC and 20ºC. However, due to the effect of abuse dehydration of the samples stored at 20ºC (>50% of start weight), it is not clear whether the aggregation of proteins in the top of the gels of samples after 14 days storage at 20ºC, can be explained by only a reaction with phenols.

Another experiment done with sugar beet leaves to determine the effect of dehydration on protein content and quality showed that short term weight loss, up to 20-25% of start weight, in a room with 60% RH, 20°C, did not affect protein levels in sugar beet leaves. The protein composition seemed also not affected. There was no clear indication of protein hydrolysis within this time frame and with respect to desiccation up to 25% weight loss.

In earlier studies (Bruins, 2020), it was experienced that proteins in sugar beet leaves should be extracted directly after harvest without any delay, to prevent lower protein yields. The reason for this remains unclear. Our results show that immediate extraction is not needed as there is no fast degradation of proteins in the intact leaves, when the material is kept at room temperature or colder. Also loss of turgor by dehydration of the leaves, is not influencing the proteins in the sugar beet leaves. Possible reason for finding lower yields could be that cuts/wounds affect protein stability after harvest. It is also probable that the lower protein yield upon delayed extractions, is related to lower volumes of extracted juice. The loss of turgor might influence the pressing efficiency and therefore the amount of proteins in the juice. Earlier experiments report much higher storage temperatures as leaves were just piled up without temperature control. This can also be the reason for decreased protein recovery.

Concluding, this study shows that:

- Low storage temperatures (< 10ºC) have a clear positive effect on the retention of protein levels and protein composition in stored sugar beet leaves and Italian ryegrass compared to leaving them at 20ºC.
- Proteins in sugar beet leaves were stable for a longer time than in Italian ryegrass.
- The speed of degradation at a temperature of 20ºC and upon desiccation is rather a matter of days or weeks, than a matter of hours as shown by this study. So the reason for finding lower protein yields, when not extracting within a few hours after harvest, is not because of fast degradation after harvest. Potential other reasons are described.
- Colour change of the biomass during storage might be a way to monitor protein levels. This needs further investigation.
- Further research would be recommended to investigate the polyphenol content (per g FW) changes and interactions with proteins in biomass, upon storage at higher temperatures and longer period. It may be an interesting field for further research in relation to the lower protein levels also found at higher temperatures.
- To optimize logistics for protein recovery from herbaceous biomass, it is recommended to study the effects of storage conditions on protein retention.

1 Introduction

The growing global population will demand 30-50% more protein in the coming 20-30 years. To be able to meet this growing demand within planetary constraints, a transition to the use of more plant-based proteins in both food as well as animal feed is essential. Furthermore, to prevent negative effects of intensive production on land and water use, deforestation, and soil erosion, especially in vulnerable areas like the Amazon, it is important to stimulate development of more sustainable supply chains of high quality (non-allergenic) proteins in Europe. This project supports the ambition of the Dutch government to only use plant-based proteins produced in Europe to feed dairy cattle by 2050.

Development of robust supply chains of plant based proteins in Europe, requires the use of new protein crops, side streams of agricultural food crops, or non-food crops (water side plants, grass). A current blind spot within these supply chains, is the possible impact of post-harvest conditions on the protein yield, quality and functionality of fractions or isolated proteins. There are sound indications that the timing of harvest, handling, storage and transport conditions of the raw material affect the final yield and quality of the different proteins (e.g. RuBisCo) present in the products. However, no systematic investigations into this issue have been done.

This project represents an exploration into the potential impact of post-harvest technology to protein supply chains. This report presents the results of a first exploration of the effects of post-harvest conditions in various types of crops on protein retention and quality. Chapter 2 describes background information on plant-based proteins collected via literature and via interviews with experts in the industry. This information was used to set up explorative experiments, to get better insights in the effects of temperature, time and water loss on protein features. Chapter 3 describes the experiment using Italian ryegrass, Chapter 4 and 5 experiments using sugar beet leaves. Chapter 6 ends with the overall conclusions and discussion.

The research was performed independently by researchers from Wageningen Food & Biobased Research, funded by the Ministry of Agriculture, Nature and Food Safety by DFI- R&D budget, within the strategic WUR-KB theme of Healthy and Safe Food. The project brings researchers with different expertise together. With the conclusions and recommendations described in this report the researchers hope to be able to indicate the importance to further develop this new field of expertise in plant-based protein research and to create substantial improvement in protein yields by innovative solutions.

2 Background information on plant-based proteins and protein extraction

2.1 Basics of plant-based proteins

Proteins are very important molecules for organisms, available everywhere in nature in diverse shapes and forms. They provide structure and function of living cells. A wide range of functions can be attributed to them. They can serve as:

- process regulators (hormones, gene expression, antibodies for the immune system);
- enzymes (catalytic effects);
- stabilizer of cell walls, skin, and bone and;
- organizers of transport of materials
- and as carriers for energy storage.

The principal structural components are polypeptide chains. These may be combined with fats as lipoproteins and with polysaccharides as glycoproteins. Proteins are complex structures. The composition of amino acids determines the three dimensional structure (helices, betasheets). And also subunits are linked together in specific ways. Molecular weights of proteins vary from thousands to millions Dalton. The molecules may consist of one single chain or two or more polypeptide chains joined by disulfide bonds. Globular proteins consist of chains tightly intertwined to form a nearly spherical shape. In some more complex proteins these spherical units may themselves be joined together into larger structures of fairly precise form.

Proteins can be found in agro-materials, plants and animals. They play an important role in the diets of animals and humans. For food consumption examples of protein rich crops are cereals like wheat, barley and sorghum and legumes like green peas, lentils, beans and chick peas.

2.2 Protein content, yields and purity in different crops

There are many sources for plant-based proteins which can be used for extraction of protein fractions or isolates and which may be used as food or feed. Globally, the protein crops with highest production volumes are maize, wheat, rice, potatoes, and soybeans (FAO datasheets, 2013). The protein content of these crops varies from 36% for soybeans on the high end to 2% protein for potatoes on the low end. Despite the protein content of potatoes is low relative to other crops, their high yield per hectare makes them an efficient protein source. Still, soybean remains the highest-yielding widely-cultivated protein crop, contributing to its position as the most economic plant-based source.

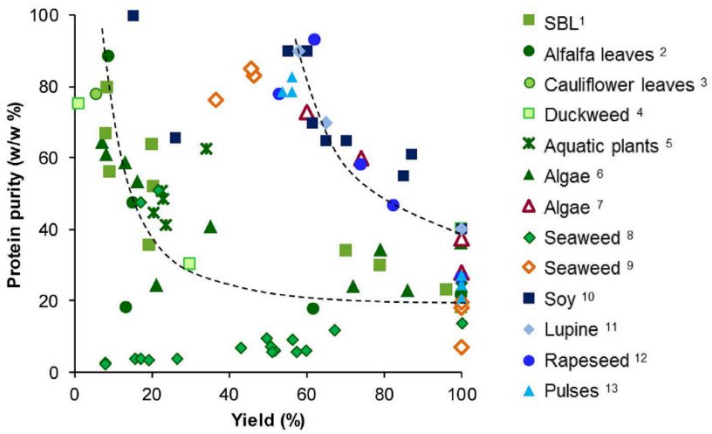

Fig. 3. Yield of extracted protein as a function of the protein purity (w/w%, dry basis) (g protein in product per gram of protein in raw material). SBL - Sugar beet leaves, [1](Kiskini, Vissers, Vincken, Gruppen, & Wierenga, 2016; Tamayo Tenorio et al., 2017a), [2](Edwards et al., 1975), [3](Xu et al., 2016), [4](van Krimpen et al., 2013), [5](Dewanji, 1993), [6](Cavonius, Albers, & Undeland, 2015; Gerde et al., 2013; Schwenzfeier et al., 2011), [7](Sari et al., 2013), [8](Angell et al., 2017; Harnedy & FitzGerald, 2013; Kadam, Álvarez, Tiwari, & O'Donnell, 2016), [9](Wong & Cheung, 2001), [10](Campbell & Glatz, 2010; Sari et al., 2013), [11](Berghout, Pelgrom, Schutyser, Boom, & van der Goot, 2015), [12](Dijkstra et al., 2003; Sari et al., 2013), [13](Boye et al., 2010).

Figure 1: Relation between yield and purity (Tamayo Tenorio et al., 2018)

Protein products are available at a wide range of purities, from flours (30-40% protein) to isolates (>90% protein). As purity increases, yield drops, as shown in Figure 1 (Tamayo Tenorio et al., 2018). Typically storage proteins, those present in relatively low-moisture environments, can be more easily isolated leading to a higher yield at a given purity. Enzymatic and membrane proteins are more challenging to extract and follow the second, lower curve.

Legume proteins, including oil-rich crops, like soy and pulses like pea, are storage proteins. The combination of high yield per hectare and relatively high and easy to obtain extraction efficiency make soy protein the most economically competitive plant protein today. Pea and faba are the highest interest pulse crops: pea due to its already-existing wide cultivation and faba due to its high yield per hectare in Europe. Ongoing research into pea and faba proteins focuses on breeding programs and conversion into appealing food products[1,2,3]. Improvements to processing efficiency are the primary focus of many commercial parties. To-date no research has studied the link between post-harvest conditions or treatments, process efficiency, and eventual protein yield.

Green plant biomass side streams are typically similar to potato in protein content on a fresh weight base, with about 2-3% proteins. Some specific leafy crops like moringa and duckweed have higher protein amounts, 6-7 and >15%, respectively. Still, green plant biomass with low protein content are interesting, because they can have high protein content on a dry matter basis. Furthermore, large volumes of green leaf material from side streams of carbohydrate crop cultivation like sugar beet are currently being unused. These proteins are clearly of interest to the industry; research has shown that they have high functional potential ((Martin et al., 2019)). For instance, one of the most abundant proteins in green leaves (up to 50%) is RuBisCo (Ribulose-1,5-bisphosphate carboxylase oxygenase) which acts as an enzyme to catalyze the first step in CO_2 fixation of the photosynthesis process. RuBisCo has interesting functional properties when not denaturated and it contains many essential amino acids (comparable with soy-protein).

Research on green leaf protein to-date has focused on extraction of protein from the leaf material ((Makkar and Becker, 1996) (Zhang et al., 2015) (Ghaly, 2010)). Extraction is an essential step in creating a strong business-case for new proteins, and especially critical when protein content is relatively low. However, the quality of protein resulting from any extraction process depends on the quality of the protein prior to extraction. In our interviews, some companies indicate that they have experienced significant decreases in protein yield when there is more time between harvest and processing.

2.3 Proteins post-harvest (before processing)

After harvest, loss of protein (quality) can occur due to proteolytic breakdown, denaturation of protein and through oxidative processes. In literature certain harvest parameters and processes in the leaves are described which influence the protein content and quality, the potential yield and/or the extractability. Here, we describe some of these aspects.

2.3.1 Dark-induced senescence

When (harvested) plants are placed in darkness, a degradation process starts, called "dark-induced senescence". One of the first organelles to undergo degradation is the chloroplast. RuBisCo, the major protein in green biomass, is present in the chloroplasts and therefore very vulnerable to degradation after harvest. During dark-induced senescence, chlorophyll-protein complexes and other proteins are degraded to smaller protein fragments, amino acids and ammonium (proteolysis = hydrolysis of proteins). This process is regulated by a diversity of proteases and protease inhibitors and is highly

[1] https://www6.rennes.inra.fr/igepp_eng/Research-teams/Resistance-and-Adaptation/Projects/Pea-Must
[2] https://has.nl/en/nieuws/new-consortium-starts-pulse-project-aimed-protein-transition
[3] https://www.wur.nl/en/Research-Results/kennisonline/AF16011-Plant-Meat-Matters.htm

dependent on stress factors (like damage and dehydration), and conditions like temperature and storage time (Diaz-Mendoza et al., 2016). Several different types of proteolytic processes and different types of proteases are involved. Often plants with high content of proteins are also more sensitive to hydrolysis (Benchabane et al., 2008).

2.3.2 Location of proteins in the crop

Several studies indicate that the sugar beet leaf blade has higher protein content than the leaf stem (or leaf blade + stem) (Tamayo Tenorio, 2017).
Benchabane et al. (2008) aimed to improve yield and quality of recombinant proteins recovered from plant protein biofactories, by minimizing proteolysis. They indicate that depending on where in the plant and/or in which organelle in the cell a protein is synthesized, proteins are more or less sensitive to proteolysis (Benchabane et al., 2008). For example:

- Proteins in leaves of plant: a high production rate of proteins but also a high protease level (involved in the senescence of the leaves). Still for production of recombinant proteins a leaf-based system is generally used.
- Proteins stored in seeds: more stable protein content as seeds during dormancy have a low water content and a low protease content.

2.3.3 Effect of crop age and harvest moment on protein levels

Leaf/crop age influences the chemical composition of leaves, including protein content (Kiskini et al., 2016). For instance Chaves et al. (2006) investigated the changes in composition when *Lolium perenne* (ryegrass) grows to maturity, to understand how digestion processes of animals fed with the grass are influenced by the stage of grass maturity. The crude protein concentration (g/kg DM) is declining upon ageing of the grass (Figure 2). The changes are associated with increases in fiber fraction and a different leaf/stem (or inflorescence) ratio. The rate of decrease upon aging was not significantly affected by mowing date.

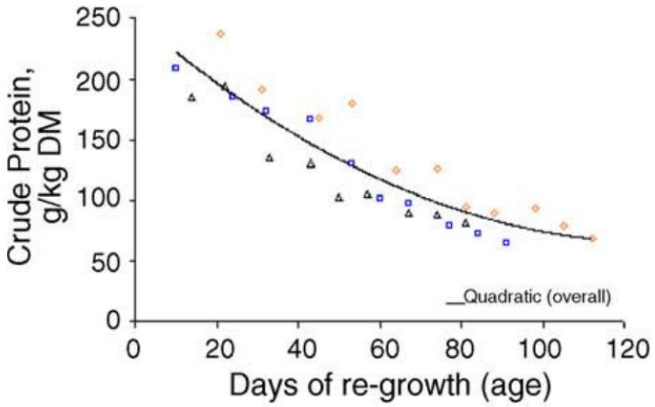

Figure 2: Crude protein composition of ryegrass, initially mown at 3 different moments (1 ◊, 2 □, 3 Δ.. Between the first and last mowing there was 1 month difference. (Chaves et al., 2006)

Keijsers (2018) has done experiments with biorefinery and protein recovery from waterside plants such as Urtica, Hydrocotyle, and Elodea. At different moments in the season samples were taken. The composition of the raw material showed higher protein content (% of dry weight) for young/early season Urtica, Hydrocotyle and Elodea, compared to late season harvests. The dry weight percentage only slightly differed between seasons. For the younger crops the proteins were located relatively more in the juice than in the fiber fraction, compared to the crop harvested later in the season.
A possible reason for the difference between the harvests is the presence of more roots later in the season, or presence of less soluble proteins later in the season (Keijsers, 2018).

Keijsers (2018) also looked at the effect of waiting 1 day after harvest (temperature not reported) with the protein extraction of various waterside plants. Visually some of the plants were clearly observed as less fresh due to the delay in processing, but the protein content (using Kjeldahl method, so actually measuring total N content), was little affected.

Studies on sugar beet leaves showed total protein content in sugar beet leaves is rather stable during the root harvesting campaign between different harvests. According to Tamayo Tenorio (2017) the leaves did present physical differences as a result of climate conditions, but their composition showed only slight variations over the harvesting campaign (Figure 3). According to Kiskini et al. (2016) variation in final protein isolation yield was mostly due to variation in nitrogen extractability, although no consistent correlation with plant age was found. A significant effect of plant age was observed here on the quality (colour) of the extracted protein, that is, brown (indicative of polyphenol oxidase activity) for extracts from old plants, compared to yellow from younger plants.

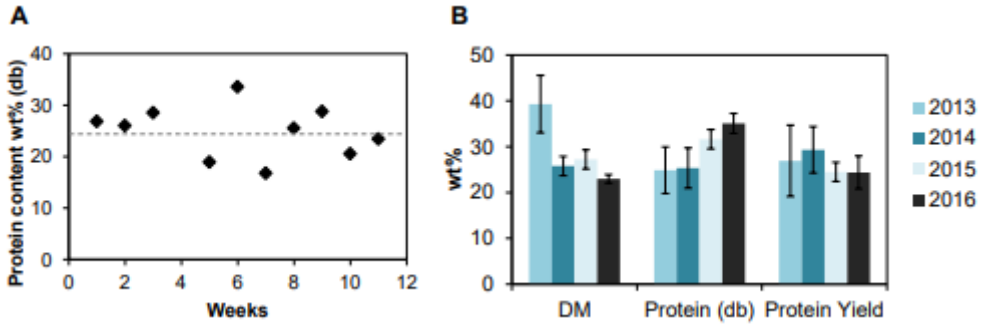

Figure 3: Composition of green pellet produced from sugar beet leaves with standard extraction process (A) protein content over 11 weeks of sugar beet's harvesting campaign. (B) Average composition (dry matter and protein) and yield of four harvesting seasons. (Tamayo Tenorio, 2017)

2.3.4 Effect of conditions after harvest on proteins

The application of silage making is an example of using fresh leafy material which is focused on minimizing nutrient losses of the grass crop and preserving the chemical composition. Effective ensilage systems aim to minimize the activity of plant respiratory and proteolytic enzymes for instance by a rapid decrease in pH or increase in DM content in the first few hours following cutting (Mayne and Buchanan-Smith, 1999). This may lead to a quick stable fermentation in the silo with limited losses of proteins.

2.3.5 Choices in logistics

It is important to realize that currently most of the green biomass which could be used for protein extraction is not grown for this purpose. Often it is a side stream at harvest time of crops, a stream of waste at a fresh-cut company or it is a waste stream of weed managing activities. The logistics part in these chains is not optimized for these streams to valorize the biomass optimally, including maintaining optimal protein content or assuring maximum protein yield.

Tamayo Tenorio (2017) has shown that for sugar beet leaves a decentralized pressing system (at the farm) combined with cooled transport of the juice to a processing facility, would be a better option than centralized pressing. Main reason is that especially transport load is reduced (pack density 73kg/m3 sugar beet leaves vs 1000kg/m3 juice, 19 trucks of leaves versus 1 truck of juice), and the remaining pulp could be directly used at the farm. But centralized pressing of the leaves would allow the use of the pulp for food or non-food uses, and possibly returning only the unused fractions to the farms; whereas processing at the farms would allow for the direct return of the pulp to the land, but

would make exploitation into useful products more difficult. Decision on the need for a freezing step of the leaves is now dependent on capacity of pressing machine. Tamayo Tenorio (2017) indicates that freezing whole leaves and then thaw before pressing has a negligible effect on the processability of the leaves and the resulting protein content and yield.

2.3.6 Conclusion

Harvest time of leafy materials may be of interest for protein yield; especially older crops may yield less protein which, in addition, may be of a lower quality. After harvest, loss of protein (quality) can occur due to proteolytic breakdown, denaturation of protein and through oxidative processes. Postharvest conditions and time in the chain may therefore affect protein yield. Also quality of the extracted protein may be affected by the postharvest conditions due to increasing oxidative stress.

2.4 Processing methods

There are many different methods of extraction and purification of proteins from diverse crops and they are dependent on many factors. Optimizing of these processes to get higher yields, higher purity, improved protein functionality and more sustainable methods, has the ongoing attention of companies and researchers.

We will describe here a few examples of reported processes for different crop types and end products to give an idea of the general concept of the process.

2.4.1 Herbaceous biomass

The proteins in herbaceous biomass can roughly be divided in 3 groups: soluble proteins and membrane bound proteins and to a lesser extent structural proteins. The soluble fraction consists of ~50% of RuBiSco. Protein products from leaves are usually obtained by pressing or shearing the fresh crops, followed by heat coagulation of the proteins, followed by centrifugation and drying (Figure 4). Because of the coagulation process that is performed at elevated temperature or acidic pH, the proteins loose most of their functional, but not their nutritional properties (Sari et al., 2015). Compared to other crop types, for leafy materials more effort is involved in releasing the proteins from the biomass (Mulder, 2016). Often more complex and energy consuming pre-treatment and isolation processes have to be used. More research is still needed to optimally extract protein from leafy biomass for high protein yields (Sari et al., 2015).

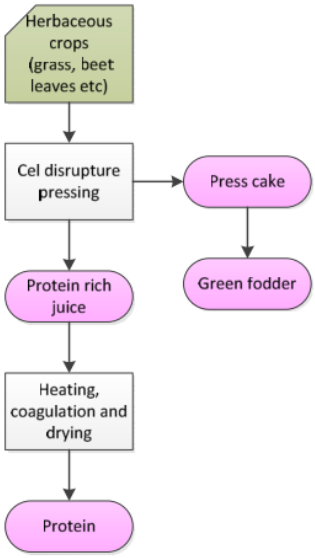

Figure 4: Herbaceous biomass refinery scheme (Mulder, 2016)

Sari et al. (2015) reviews the addition of various alkali (hydroxides) in the extraction process. This is done to increase protein extraction yields compared to extraction with only water. Also less soluble proteins can then be extracted. Different types of alkali and sometimes combinations are used to extract protein from several types of leaves. Examples of alkali which are being used are NaOH or $Ca(OH)_2$, and ammonia.
In some cases enzyme addition, especially of proteases, can be useful when alkaline protein extraction yields are low. Proteins will be reduced in protein size which facilitates the extraction. These additions can also be used to enable processing at a pH closer to 7 to avoid the otherwise severe conditions that denature proteins. This method needs further optimization.
Specific protocols are developed to isolate RuBisCo from proteins, of which several are patented. All methods start with a disruption method to release the juice. As this juice is prone to oxidation, reducing agents can be added to prevent discoloration. This is only mentioned in the WFBR patent (WO2014104880), but currently also investigated by other groups (e.g. Aarhus University). The method described in the WFBR patent is currently developed further towards commercialisation by COSUN in the Greenprotein-EU project. Subsequently two approaches are then used;
1) Heating of the juice, as RuBisCo is relatively stable around 60°C and a solid agglomerate of the chloroplastic proteins, chlorophyll, carotenoids, and lipids can then be removed or 2) Addition of polyethylene glycol (PEG) to precipitate or crystalize RuBisCo by lowering its solubility (Bruins, 2020).

2.4.2 Pulses/legumes

Pulses (including peas) are an interesting source of plant proteins with a high initial protein content. Pea contains around 24g protein per 100g dry matter (Schutyser et al., 2015). The main storage proteins in legume seeds are globulins (legumin and vicilin) and albumins (Karaca et al., 2011). To extract proteins from starch-rich legumes, such as peas, a wet route is commonly used, in which legumes are dispersed into water to hydrate and solubilize the protein and suspend the starch granules. Subsequently the slurry is treated in a decanter to separate the proteins from the starch granules and the fibres. The albumins and the globulins are separated by isoelectric precipitation (pH 4.2 to 4.8) using a decanting process. The pH of the precipitate (mainly globulins) is increased to pH 7-8 before pasteurization/sterilization. In a next step, a dry protein isolate is obtained after spray drying step (Schutyser et al., 2015) (Figure 5). An alternative described for this wet process is dry fractionation (Schutyser et al., 2015), but this method leads to lower protein purity and no separation between albumin and globulins. However, due to the mild processing, the proteins are still in their native form, which may have an effect on the functionality and digestibility of the final ingredient. The final product is a protein concentrate.

Figure 4

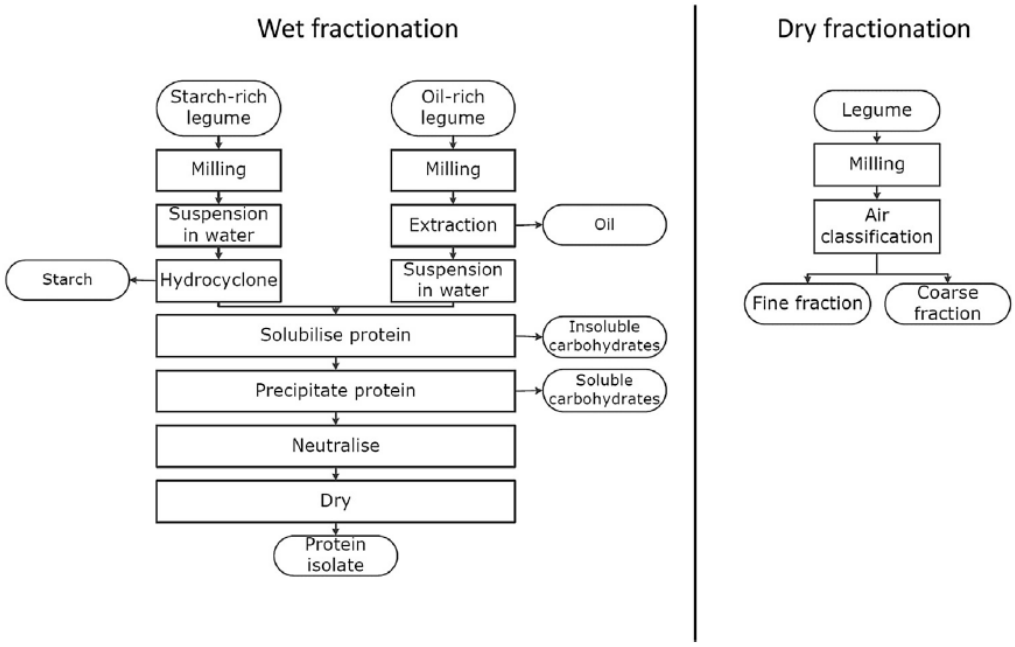

Figure 5: Schematic illustration of wet (left) and dry (right) fractionation process of legumes (Schutyser et al., 2015)

2.4.3 Other crops

In many crops, the protein is not the main product of interest, but starch or oil is. First oil or starch is being extracted, followed, only then followed by a protein step. These are excluded from our focus in this study as the protein content and quality is often already influenced by the first extraction step targeted for the primary goal. (Mulder, 2016) provide in their report clear schemes of the typical processes involved in processing of these crops. Currently there are developments for some crops in the industry to switch from primary extracted product. For instance, there are developments for potato crop, normally with starch as primary product, to switch to proteins as primary product, followed by other refinery steps (Figure 6).

Figure 6: Example of an advanced potato refinery process (Solanic) producing proteins for food applications [Wageningen UR](Mulder, 2016)

2.5 Protein analysis methods

There are several ways to quantify the protein level in raw material. Kjeldahl[4] or combustion analyses[5] like Dumas[6] are often used to get a relatively fast impression of protein content. However, it is an indirect indication, via measurement of total N. These methods are not suitable to determine any protein breakdown during storage or distribution. If proteins are (partly) hydrolysed or oxidized it will not be noticed using this method. For more selective analysis methods, to measure proteins itself, often either Bradford[7] or BCA-assay[8] (bicinchoninic acid) methods are used. Disadvantage of Bradford assay is the interference with polyphenols or colour in the samples. Gel electrophoresis[9] shows the presence of proteins sorted by their size and charge. This method can show the degree by which proteins have been broken down and if target proteins such as rubisco are still intact in the samples. In the experiments described in this report we have used several combinations of methods.

[4] https://en.wikipedia.org/wiki/Kjeldahl_method
[5] https://en.wikipedia.org/wiki/Combustion_analysis
[6] https://en.wikipedia.org/wiki/Dumas_method
[7] https://en.wikipedia.org/wiki/Bradford_protein_assay
[8] https://en.wikipedia.org/wiki/Bicinchoninic_acid_assay
[9] https://en.wikipedia.org/wiki/Gel_electrophoresis

2.6 Research priorities

Based on the outcome of the collected background information in Chapter 2, the following research priorities for experiments in this project have been determined:

- Gaining insight in the effect of temperature and time conditions after harvest on protein retention and on Rubisco levels in various green herbaceous crops (grass and sugar beet leaves as key crops).
- Gaining insight in the effect of raw material dehydration and wounding after harvest on protein retention and quality (sugar beet leaves as key crop).
- Gaining insight in the effect of storage conditions on protein retention and quality in pulses (yellow pea as key crop).

Experiments and results with sugar beet leaves and grass are described in the following chapters.

3 Effects of storage temperatures and duration on proteins in grass

3.1 Introduction

Grass was selected as crop to be used in the first experiment. It is a potentially interesting biomass source from "waste material", containing among others Rubisco. Moreover, the choice for grass was made because of the availability and the option to have control over the harvest and handling after harvest. Goal of the experiment is to gain insight in the effect of temperature and time conditions after harvest on proteins in grass. Specific attention goes to the total content of proteins and on potential breakdown or loss of functionality. We will focus on Rubisco levels and breakdown of proteins in smaller molecules.

3.2 Set-up

Grass (Italian ryegrass *Lolium multiflorum*) was grown at Unifarm (Wageningen, the Netherlands, parcel 10) during the spring of 2019. At May 20th grass was harvested from the field, using a Haldrup mower. The grass was transported in plastic bags to Wageningen Food & Biobased Research (Wageningen) (Figure 7).

Figure 7: Collection of the plant material at Unifarm, Wageningen, NL

3.2.1 Sample preparation

Samples were prepared by filling white plastic boxes of 1 litre with 50g of grass. Each box was closed with a lid with 5 holes, prepared using a thick needle. Samples were stored in climate rooms at several controlled temperatures (20, 10, 5, 1, -1°C) and 80% RH. At regular intervals, temperature was logged from a selection of boxes, dispersed in between the samples for other analyses.

3.2.2 Weight loss

Boxes were (pre)-weighed using Mettler 6002 2017-02 scale (Figure 8a). After filling, the total of box and sample was weighed. After storage the box and sample were weighed one more time and weight loss was calculated.

3.2.3 Dry matter

Per sample approximately 10 grams of grass was put in a (pre-weighed) Pergamon paper bag and weighed, using a Mettler Toledo type MS403TS/00 scale. The bags were placed at 80°C and weighed again after 4 days. Dry matter content was calculated.

3.2.4 Colour images and analysis

Colour images of the grass samples (3 replica's) were taken in the box, without lid, under standardized circumstances in a cabinet mounted with LED arrays on 5 sides (4038 K), designed by WFBR and built by IPSS Engineering (both Wageningen, Nederland). The cabinet is equipped with a RGB camera (MAKO G-192C POE, Allied Vision Technologies GmbH, Stadtroda, D) which takes images from above using standardized settings. (Figure 8c). Prior to each measuring session the system is calibrated using a white background (Forex® PVC sheet white 6mm) 24 squared colour cart (Colour checker classic, X-rite Europe GmbH, Regensdorf, S). Based on this calibration, the RGB images are transposed to the official L*a*b* (D50) values of the Macbeth ColourChecker (Pascale, 2006).

The Colour Learning software (developed by WFBR) was used to teach the grass colour class for classification. Out of all images, extremes were selected to feed the Colour Learning software. After defining the colour class, from each sample the average L*, a* en b* and ΔE, were calculated in the Colour Analysis software (developed by WFBR).

3.2.5 Sample preparation for protein extraction

On each evaluation day and for each treatment, 5g of grass was sampled and stored at -80°C till protein extraction and Kjeldahl analysis (Figure 8b,d).

3.2.6 Protein extraction

Frozen grass samples were ground using an IKA analytical mill (IKA-A11-Werke GmbH & Co., Staufen, D). 100 mg of frozen material was weighed, 475 µL of protein extraction buffer (Agrisera AB, Vännäs, Sw) supplemented with protease inhibitors (Complete®; Roche, Basel, CH) was then added together with a 3 mm tungsten carbide bead to assure complete cell tissue disruption during shaking. The sample was shaken in stop steps of 2 min at 30Hz with an MM301 Vibration Mill (Retsch GmbH, Haan, D). Samples were then centrifuged at 10.000 x g for 5 minutes. Supernatant was carefully collected and transferred into a new Eppendorf tube. Supernatant was one more time centrifuged at 10.000 x g for 5 minutes to discard the remaining cell tissue. Supernatant, containing the targeted proteins, was collected and stored at -20°C till analysis. During the several steps of the proteins extraction, samples were kept on ice in order to minimize protein breakdown.

3.2.7 Protein gel (SDS-Page)

Changes in protein composition can be examined with Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The stability of the Rubisco protein can be followed from the protein bands of the two major subunits, the large 53 kDa subunit and small 14 kDa subumit. SDS-PAGE was performed using a Mini Protean II system (Bio-Rad) with AnykD Mini-Protean TGX Precast gels. On each gel, MW markers (broad range of 6.5 to 200 kDa, Bio-Rad) were used as reference for protein characterization on molecular weight determination.
Thawed extracts were resuspended and diluted 1:1 v/v ratio with SDS sample loading buffer (2x) with reducing agent (0.125 M Tris-HCL buffer pH 6.8 containing 4% SDS, 20 % glycerol, 10% 2-mercaptoethanol and, 0.004% bromophenol blue). The samples were subsequently heated for 10min at 95°C in a mixing heating block and then centrifuged at 12.000 g for 5 minutes. An amount of 12,5 µl supernatant of each sample was loaded on the gel and electrophoresis was carried out at 150 V (constant) for about 1 hour.
The proteins were stained with Coomassie Brilliant Blue (CBB) R250, 0.2% in 40% methanol, 12% acetic acid solution at 35 °C for 1 hour. De-staining was done with a 10% methanol, 10% acetic acid solution at room temperature.

Gels and protein patterns were documented and analyzed with the imaging system Chemo Doc touch (Bio-Rad) and Image Lab Software 6.1

3.2.8 Total nitrogen content (Kjeldahl)

Total Nitrogen content was analysed according to the Kjeldahl method. 5 g of frozen samples were first digested using a Gerhardt Kjeldatherm instrument. Digestion occurs by placing the sample into a tube with one tablet Kjeltab and 9mL of H_2SO_4 and heated up to 420°C for 50 minutes. Distillation and titration of the digested sample was applied in a Gerhardt Vapodest 450 instrument. The titration was applied with 0.1M HCl acid solution.

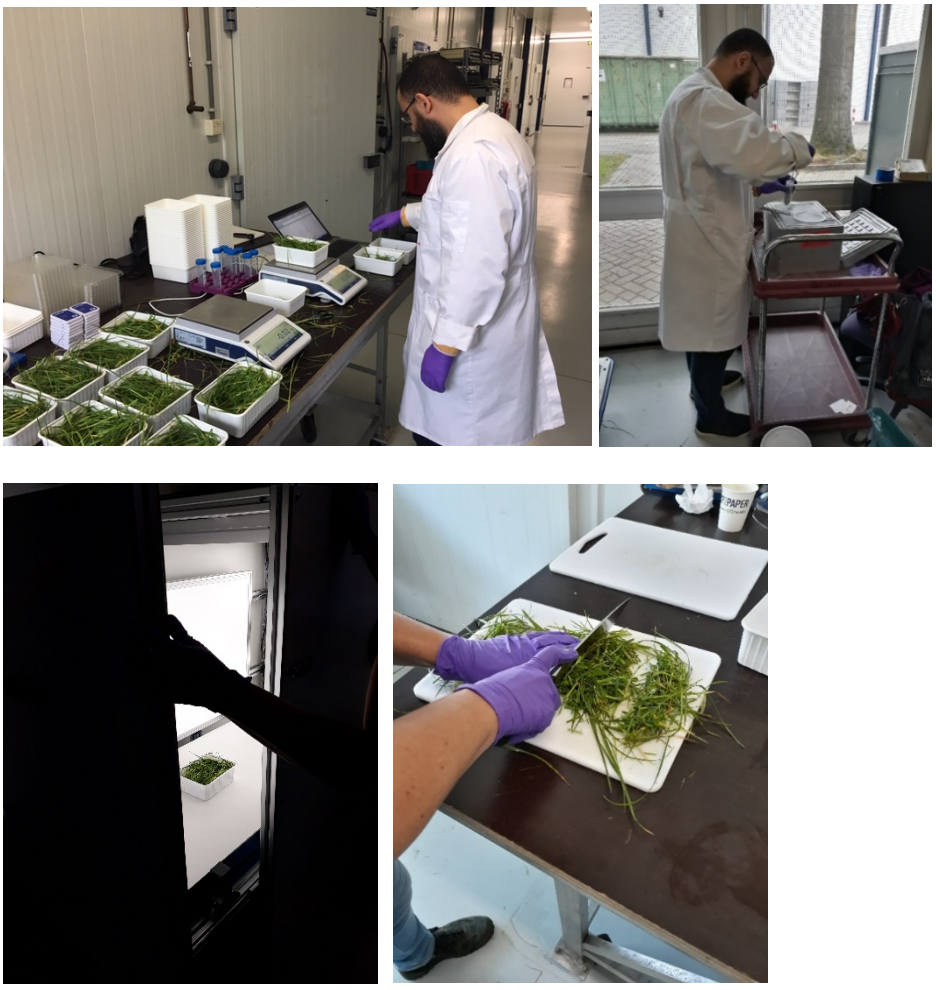

Figure 8: Impression of sample preparation. Weighing the fresh grass samples (a), freezing the samples in liquid nitrogen (b), taking a calibrated image (c) and preparing samples after storage (d)

3.3 Results

3.3.1 Appearance and colour

The pictures of the different samples show yellowing/browning of the grass in dependence of the time and temperatures of storage (Figure 9). The grass stored long and at higher temperatures also developed mould growth (≥2 weeks 20°C). By the naked eye, clear yellowing is seen after 3 days at 20°C and after 7 days at 10°C. At -1°C virtually no yellowing is observed for the duration of the experiment (22 days).

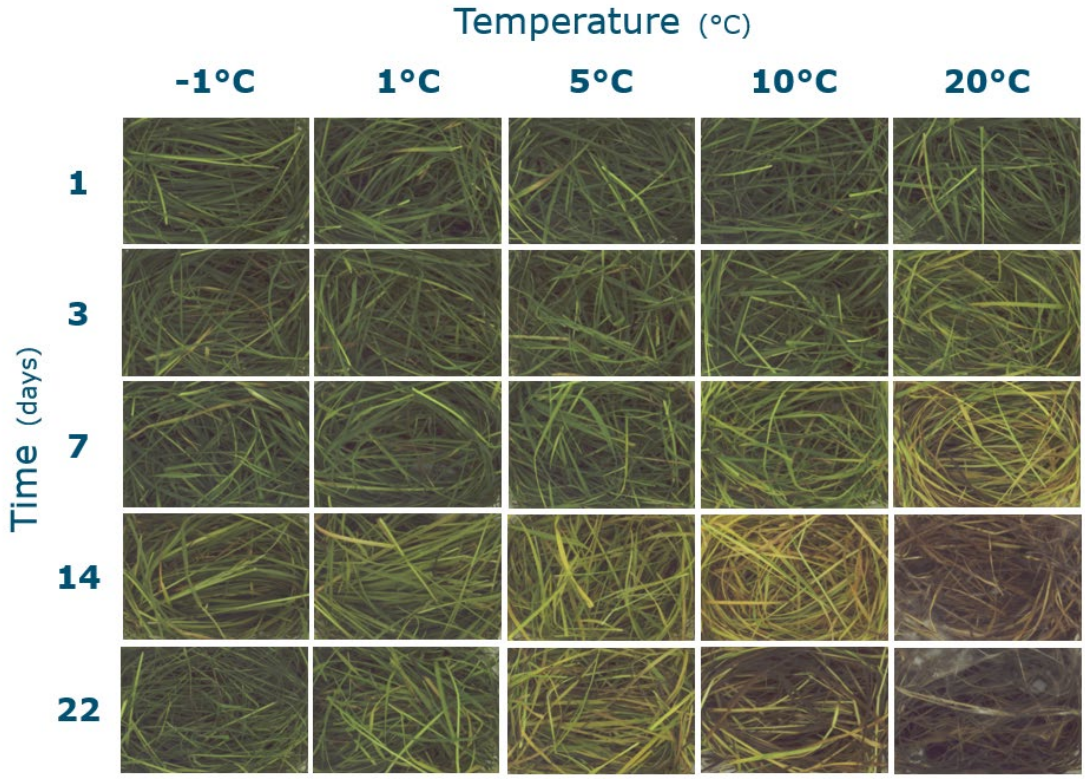

Figure 9: Matrix with impression of colour of grass samples (calibrated pictures) after different storage periods and different temperatures.

Quantitative analysis of the different colour components in the images is presented Figure 10. The analysis of colour and colour changes could be informative for what happens to the proteins in the samples.

The relative colour change, Δ E (calculated change of colour based on change of L*, a* and b*) in the sample between day zero and day x) is given in Figure 10. This shows a rapid change of colour especially in samples stored at 10 and 20°C; a slower change of colour in samples stored at 5 and 1°C and no change of colour in samples stored at -1°C. The dynamics of Δ E closely resembles the visual judgement.

Value a (Figure 11), depicting the change in colour between green and red shows a clear increase (more red) over time for grass stored at 20 and 10°C, a smaller increase for grass stored at 5°C and no increase for grass stored at 1 and -1°C. The dynamics of value a closely resembles the visual judgement of the photos in Figure 9.

L (lightness, Figure 12) and b value (from blue to yellow, Figure 13) both show a slight increase during the first days of storage for samples at 10 and 20°C, with a sharp decrease only at samples stored at 20 and 10°C.

These results show the potential to use colour change (Δ E) to describe visual quality of stored cut grass.

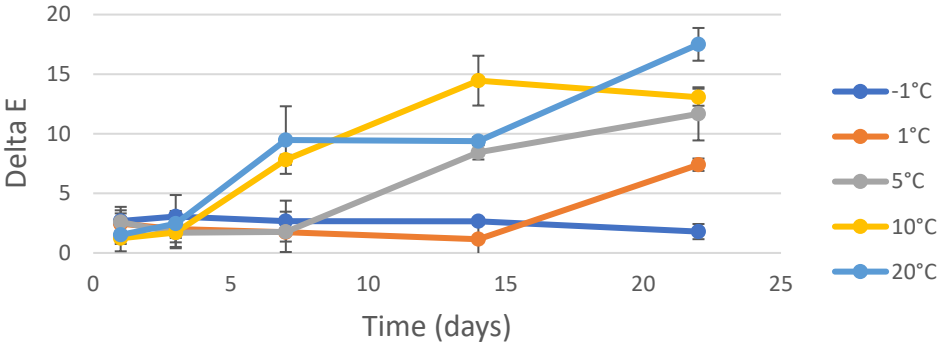

Figure 10: Colour change ΔE in time with temperature, measured in independent samples, and the 95% confidence interval (n=3)

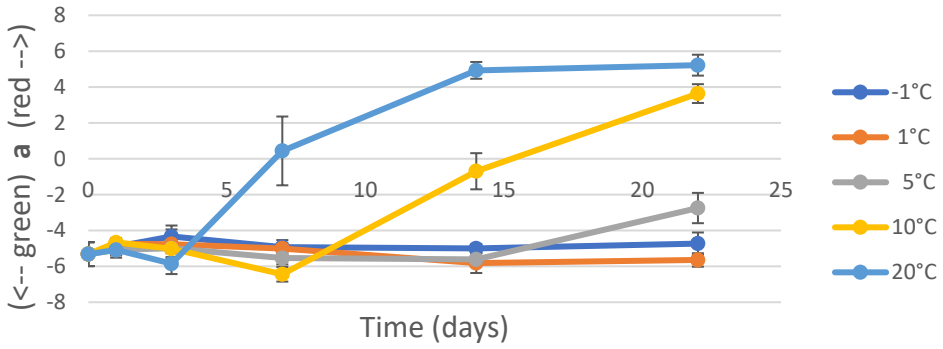

Figure 11: Colour component a in time with temperature, measured in independent samples, and the 95% confidence interval (n=3)

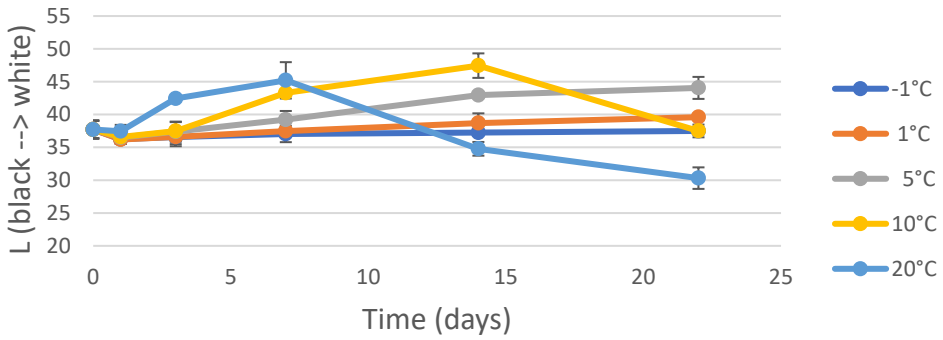

Figure 12: Colour component L in time with temperature, measured in independent samples, and the 95% confidence interval (n=3)

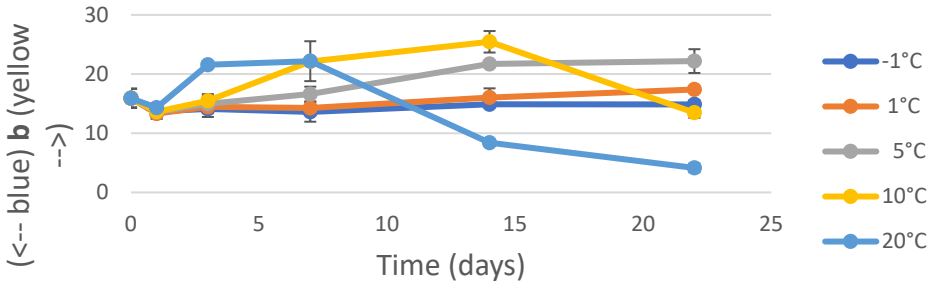

Figure 13: Colour component b in time with temperature, measured in independent samples, and the 95% confidence interval (n=3)

3.3.2 Weight loss and dry matter development

The samples show a gradual loss of fresh weight per temperature, most severely at the highest temperature (Figure 14). For all temperatures except 20°C the fresh weight loss on average stays below 5% after 22 days, but for 20°C after 3 days already 5% was lost. The weight loss is not only related to water loss, but also to loss of dry matter, as can be seen in Figure 15.

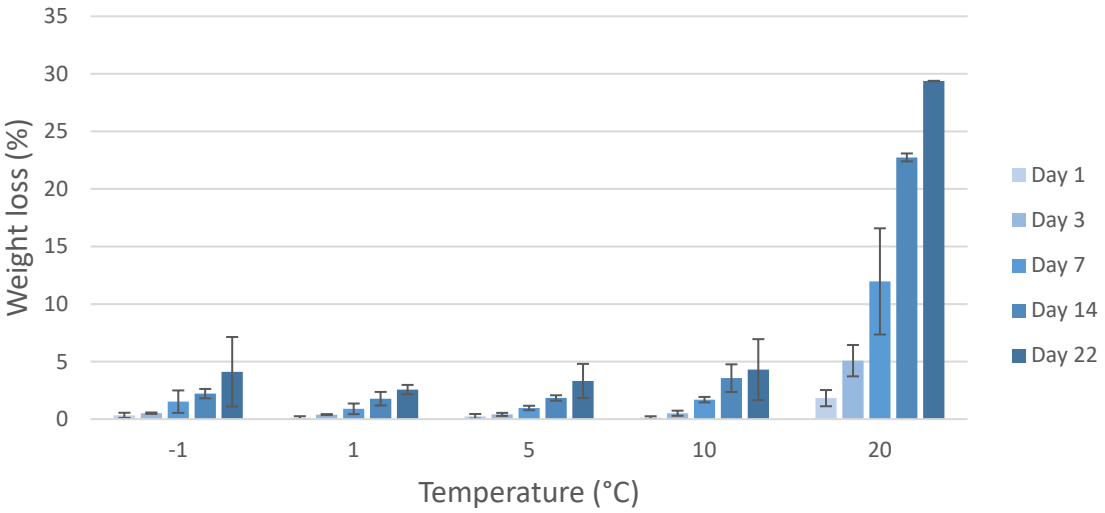

Figure 14: Percentage of fresh weight loss (compared to fresh weight at the start) at various storage durations/temperatures. Error bars indicate 95% confidence interval (n=3)

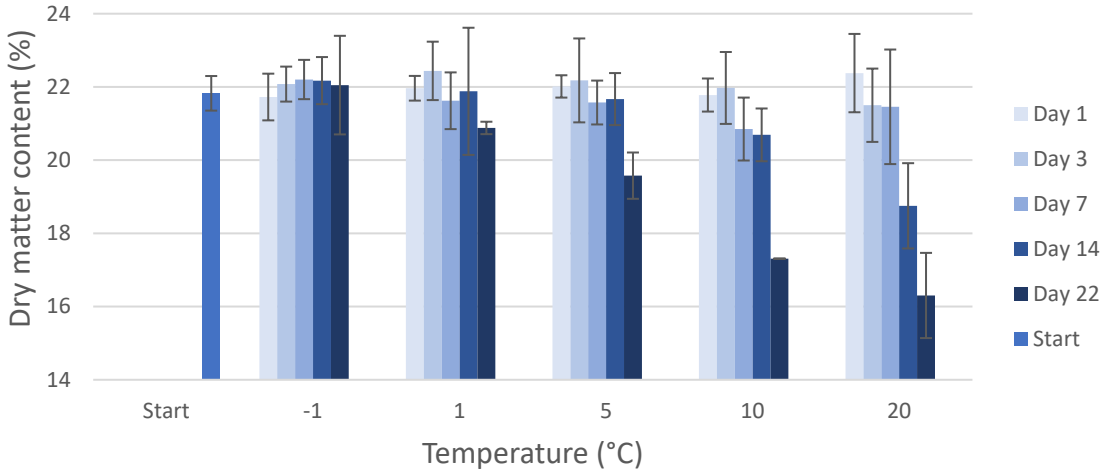

Figure 15: Percentage of dry matter at various storage durations/temperatures. Error bars indicate 95% confidence interval (n=3, except at start: n=6)

3.3.3 Total nitrogen content

The total nitrogen content was measured via the Kjeldahl method and results are summarized in Figure 16 and Figure 17. The total nitrogen content, expressed per gram fresh weight, did not show a clear correlation with the time and temperature during storage (Figure 16). On average the amount was between 3.5 and 4 mg/gFW at temperatures up to 10°C. At 20°C total nitrogen seemed to show an increase with time, up to 5 mg/gFW. This is presumably related to the severe water loss that occurs at 20°C thereby providing more "concentrated" samples. When nitrogen content is corrected with the dry matter content and expressed in mg N per g dry weight, the calculated total nitrogen content appears more constant during the complete storage period at all temperatures (Figure 17). Only grass samples stored for more than 14 and 22 days at 20°C showed a clear increase of nitrogen

content (and decrease of dry weight). Czerkawski (1967) observed similar behaviour of total N content after long storage at high relative humidity. N content of dried ryegrass increased when grass was stored at 80% relative humidity. However when expressing the N content per gram of ash, no significant effect of the relative humidity during the storage period on the total nitrogen content was visible anymore. In the present study, the ash content was not measured. The increase of nitrogen content observed in grass samples stored for more than 14 days at 20ºC, may also be explained by the high fungal growth observed on these samples. Maybe this interfered with the measurement or extraction.

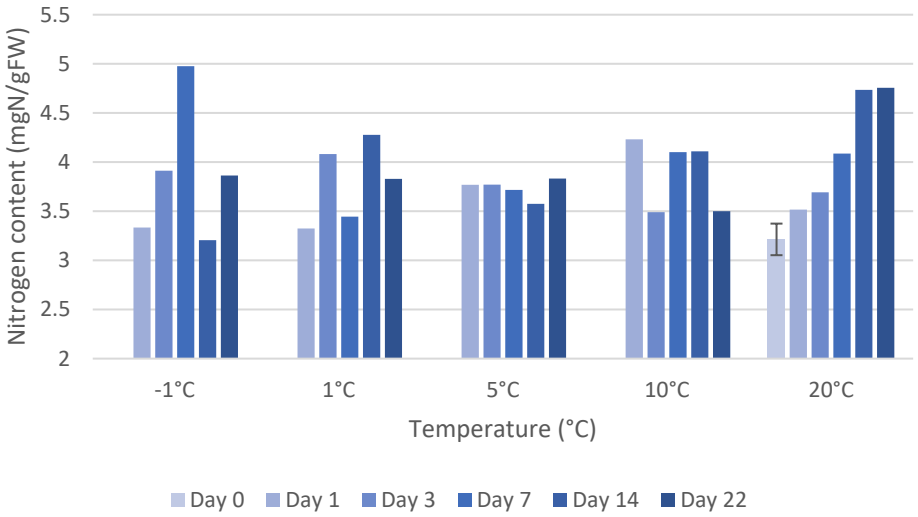

Figure 16: Total nitrogen expressed per gram fresh weight in grass samples stored for different periods at different temperatures

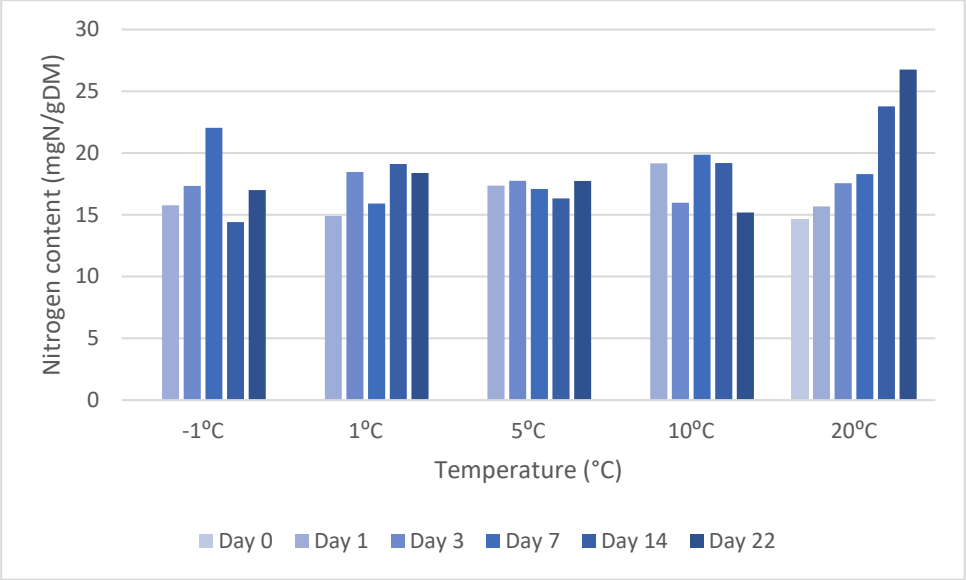

Figure 17: Total nitrogen expressed per gram dry weight in grass samples stored for different periods at different temperatures. Each bar represents the nitrogen content of one individual sample.

3.3.4 Protein (Rubisco) breakdown

Protein extracts were made from a fixed amount of frozen material; not corrected for water loss of the samples. Protein samples were run on gel (Figure 18) to judge possible protein breakdown and specifically breakdown of Rubisco in subunits. Gels clearly show the Rubisco large subunit (53kD). The other abundantly present protein (28kD) presumably represents the chlorophyll light harvesting complex. Rubisco small subunit (14kD) is not visible on the gels. The absence of the small subunit on the gel has been also observed on previous work (internal report WFBR). It seems that the extraction

method using a heat step or not may explain the presence or absence of the small subunit on the protein gel. This theory was not verified in the present study.

A clear degradation of protein is observed over time especially at the higher temperatures. At 10 and 20°C, most protein is broken down already within 7 days. At day 3, however, it seems that under all conditions (except for storage at 20°C), proteins are still intact. At 20°C, also at 3 days some protein breakdown is visible.

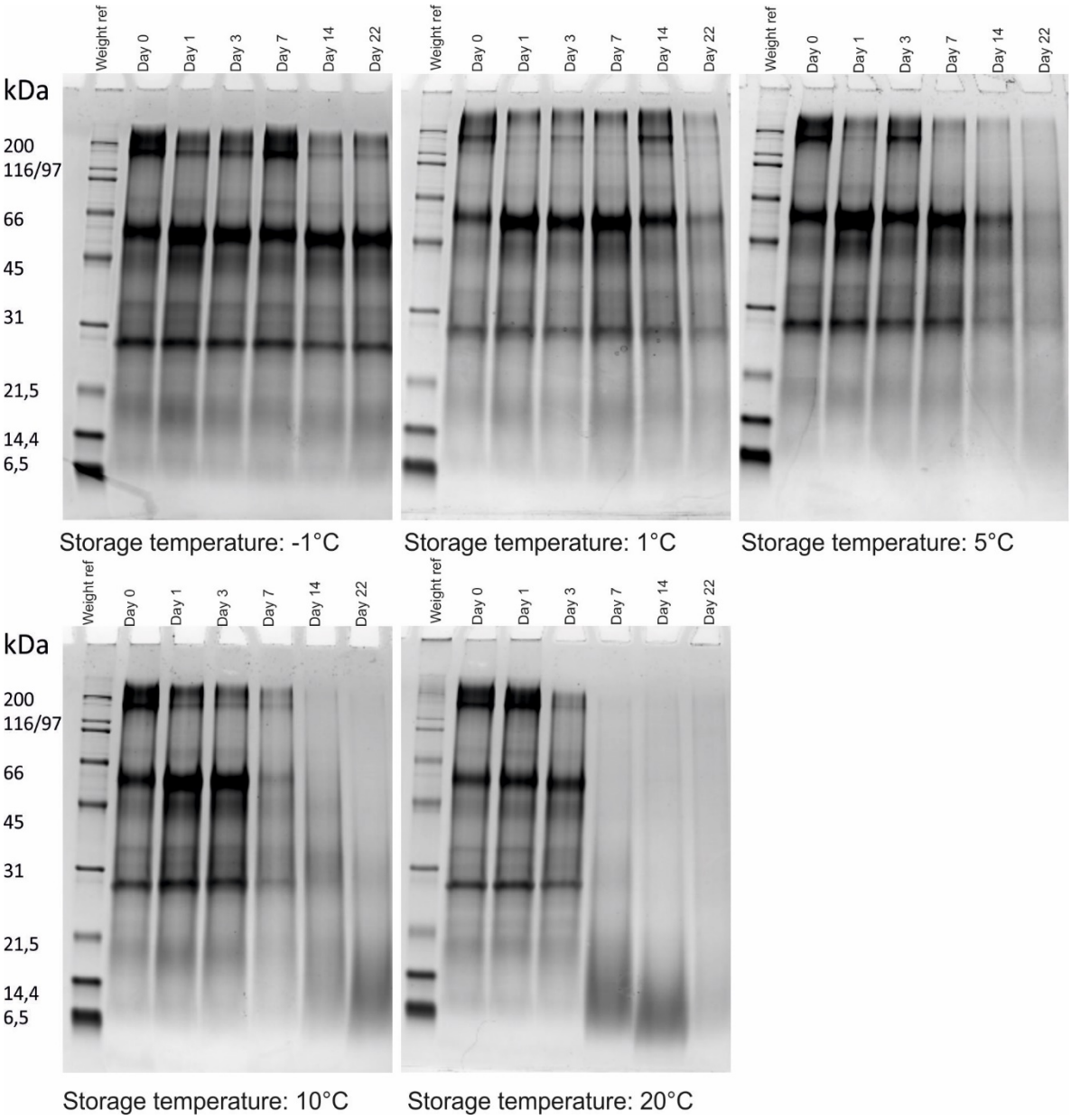

Figure 18: SDS page gels of proteins extracted from fresh grass material stored for different periods of time at different temperatures

3.4 Conclusion and discussion grass

It was seen in the experiment that there was a gradual loss of fresh weight during storage, which was most severe at the highest temperature (20°C). The weight loss was not only related to water loss, but also to loss of dry matter. This may be due to plant respiration or the presence of fungi in the samples after long storage at high temperatures may have interfered here.

The total nitrogen levels in the samples did not show a clear correlation with time or temperature. The levels remained relatively stable when plotted per fresh weight or dry weight (which was also expected). However samples at 20°C showed an increase of N content over time, which is surprising.

Proteins (specifically looking at the large subunit of Rubisco) are broken down faster at higher temperatures. At 10 and 20°C, most protein is broken down already within 7 days. At day 3, however, it seems that under all conditions (except for storage at 20°C), proteins are still intact. At 20°C, also at 3 days some protein breakdown is visible.

The measured relative colour change ΔE but also other colour related parameters (L, a, b), were quite indicative for the visual colour change in the samples. The colour change seems to occur at the same moment when protein breakdown starts to become visible. The colour is the end result of degradation/proteolysis of the chloroplast, including the proteins present in the chloroplast, like RuBisCo.

It can be concluded that low temperatures (between -1 to 5°C) can keep protein content and quality in grass stable for several days to weeks. At 10 and 20°C clear protein degradation is observed after more than 3 days. This is reflected in change of colour of the material and, at long storage times in rotting of the material.

4 Effects of storage temperatures and duration on proteins in sugar beet leaves

4.1 Introduction

End of 2019 a new experiment was started using a different crop, sugar beet leaves, to see whether the found effects of temperature/time storage conditions on protein content and stability are similar as observed in stored grass. The following hypotheses have been set up:

- Storing at low temperature slows down Rubisco depletion
- There is an increase in the phenol content when stored longer and under higher temperatures

It was expected that by storing the leaves at low temperature, enzymatic and physiological processes would be suppressed/slowed down, resulting in better retention of protein and other nutrients. This effect was investigated in raw material stored at different temperatures. Theoretically, storage may affect the efficiency of protein extraction in industrial processes. This was not taken into consideration. Proteins were extracted from frozen material with presumably high efficiency and independent of storage conditions. We expected that during storage the phenolic compounds may have increased. This might be relevant as it can affect the quality of isolated proteins.

Sugar-beet leaves (leaves without crown) were be stored at several storage conditions (-5, 1, 10 and 20°C) for different storage durations (0, 1, 3, 7, 14 and 21 days). Following parameters were determined:

- Colour changes: pictures of bunches of leaves before processing were taken with the colour light cabinet
- Weight loss (water loss, dry weight loss)
- Protein content: BCA-analysis (on frozen leaf samples)
- Rubisco stability and protein degradation: SDS gel (frozen leaf samples)
- Total protein content (BCA) (on frozen leaf samples)
- Total Phenolic compound content (on frozen leaf samples)

4.2 Set-up

Sugar beet was grown at Unifarm (Wageningen, the Netherlands, parcel lot HH2, HH3 and HH4) during 2019. At October 15th ± 70 sugar beet leaves with the crown were manually harvested using a sharp knife. In this way the leaves were still connected and prevented from drying out (Figure 19). The sugar beet leaves were transported by car to the lab (± 10 minutes). Since the leaves were dry, no centrifuge step was necessary. Triplicate samples were made per storage time (0, 1, 3, 7, 14 and 21 days) and temperature (-5°C, 1°C, 10°C and 20°C). Per sample bunches of five leaves were made from a randomized selection of 10 crowns. Only leaves from the middle of the plant were taken, not the older and younger leaves. The bunches were weighed and placed in a crate with a polystyrene sleeve at the bottom. To fill up the crate three more dummy bunches were made and placed in the crate together with the triplicate samples.

The crates were photographed in the calibrated colour cabinet. Prior to moving the crates to the right temperature, another polystyrene sleeve was placed on top of the bunches. Once the crates were set in each cooling room, an extra plastic sheet was put on top of the staple to limit dehydration (Figure 20).

After 0, 1, 3, 7, 14 and 21 days, a crate with samples was taken from their storage at different temperatures. A picture of the crate was taken in the colour cabinet and the 3 bunches were weighed, followed by taking leaf samples for protein and phenol analysis and dry matter analysis.
Samples from the leaf for analyses consisted of the top 15 cm of the leaf tissue, divided in a left and right side, excluding the midrib. One side was frozen in liquid nitrogen and stored at -80°C till protein and phenol extraction and analysis. The other side was put into a bag for dry matter analysis.

Figure 19: Impression of harvesting and preparation of samples sugar beet leaves

Figure 20: Impression of storage treatments of sugar beet leaves. Left: at -5°C in a cabinet, right: at 1°C in a room

4.2.1 Dry and fresh weight measurements

For the weight loss determination, boxes were weighed using a scale measuring with 2 decimal digits precision (Mettler) at the beginning of the storage period and on the evaluation day. The difference of weight was divided by the initial weight and expressed in % water loss**.**
For the dry matter measurement, samples were taken as described in 4.2. Per sample sugar beet leaf (5 half tips of each leaf in a bunch) were put in a (pre-weighed) Pergamon paper bag and weighed, using a scale with 3 decimal digits precision. The samples were dehydrated for 4 days at 80°C. Dry samples were weighed again with the same scale. Three samples per condition were weighed.

4.2.2 Colour cabinet: as described in 3.2.

The Colour Learning software (developed by WFBR) was used to teach the sugar beet colour classes for classification. Out of all images, extremes were selected to feed the Colour Learning software.

4.2.3 Protein extraction

Frozen sugar beet leaf samples were ground using an IKA® A11 analytical mill (IKA®-Werke GmbH & Co., Staufen, Germany). 100 mg of frozen leaf material was weighed into a 2 mL Eppendorf tube. 650 µL of protein extraction buffer (Agrisera AB, Vännäs, Sweden) supplemented with proteinase inhibitors (Complete®; Roche, Basel, Switzerland) was added, as well as a 5 mm tungsten carbide bead (Qiagen). The sample was shaken at 30Hz for 2 * 2 min in an MM301 Vibration Mill (Retsch) and centrifuged at 10.000 g for 5 minutes. Supernatant was carefully collected and transferred into an Eppendorf tube. The sample was centrifugated one more time at 10.000 g for 5 minutes to discard the remaining cell tissue. The supernatant, containing the proteins, was transferred into Eppendorf tubes for storage at -20°C till analysis. For each analysis (SDS-Page and BCA a separate Eppendorf tube was prepared per sample).

4.2.4 Protein gel (SDS-Page)

The same method as described in paragraph Protein gel (SDS-Page)3.2.7 was used to follow the changes in protein composition. Only a different dilution thawed was used (1:2 v/v ratio) for the extract and SDS sample loading buffer.

4.2.5 BCA analysis

Total soluble protein content in extracts was measured using the modified Biuret method (Pierce™ BCA protein assay kit). The extracts of the samples (4.2.3) were thawed and diluted 20 times with saline solution (0.9% NaCl). In a 96 wells plate 20 µl diluted samples and BCA standard solutions were mixed with 200 µL of BCA Working Reagent (WR). The samples were incubated at 37°C in a thermo plate mixer for 30 minutes. Absorbance at 562nm was measured with a spectrophotometer (Versomax). The Bovine Serum Albumin (BSA) calibration curve was used to calculate the protein content in µg BSA/mL. Final results are expressed in mg (BSA) protein/g fresh weight and mg BSA protein/g dry weight.

4.2.6 Total phenol content analysis

The Total phenol content (TPC) was measured using the Folin-Ciocalteu reagent according to the method described by Witkowska and Woltering (2014), with minor modifications. The total phenol components were extracted from grinded frozen sugar leave material. 250mg of frozen material was weighed into 2mL costa tube. The extraction consisted of adding 1ml of cold pure methanol to the leave material and one 5 mm tungsten carbide bead (Qiagen). The tube was shaken for 2 times 2 minutes at 30Hz using a MM301 Vibration Mill (Retsch). Tubes were then gently shaken on ice for 1 hour at 250 movements per min. Tubes were vortexed prior to centrifugation for 10 min at 15500 g and 4ºC. Supernatant was then discarded and filtered with 0.45µm filter (Minisart Syringe filter from Sartorius, Goettingen, D). 100µl of extracted sample was diluted into 6mL demi water and 0.5mL FC reagent. Solution was left 3 minutes at room temperature before adding 3.4 mL of sodium carbonate (8.82% W/v). Tubes were directly vortexed and incubated 30 minutes at 40°C. Absorbance at 765nm was measured after incubation period with a spectrophotometer UV-3100PC (VWR,Radnor, PA).

The calibration curve was established by measuring absorbance of a gallic acid solution (0-12.5µg/mL). TPC was expressed in µg gallic acid equivalent per gram fresh weight of sugar beet leave (µg GAE/g FW)

4.3 Results

4.3.1 Colour

The pictures of the different samples do not show clear yellowing/browning of the sugar beet leaves over time or with increasing temperatures (Figure 21). The sugar beet leaves stored at -5°C were frozen en became darker. At 20°C the leaves turned lighter after 7 days and became darker after 14 days of storage due to drying out. The quantitative colour measurements of the pictures with the complete bunches were not so indicative in this experiment (interference stem/leaf), therefore these are left out of this report.

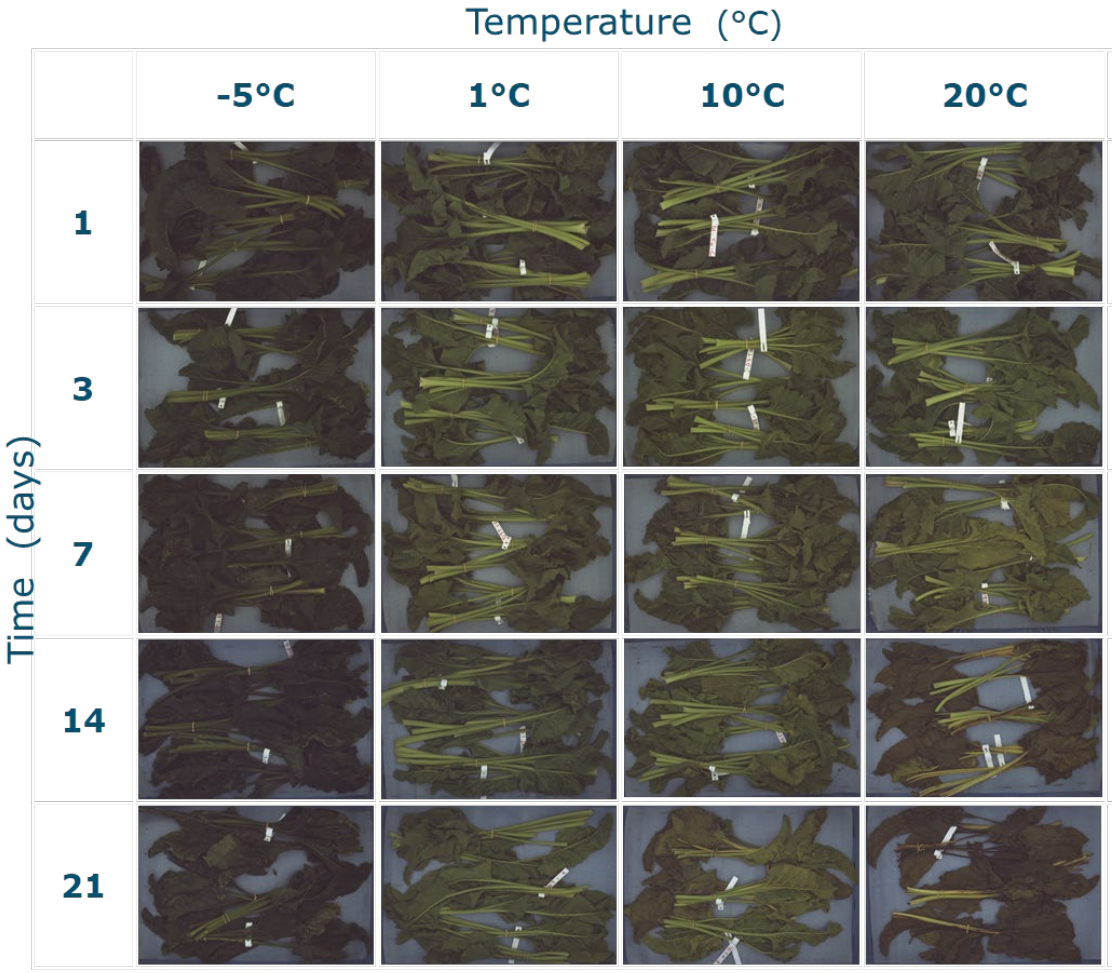

Figure 21 Matrix of calibrated pictures with impression of colour of sugar beet leaves samples after different storage periods and different temperatures

4.3.2 Weight loss and dry matter development

Over time, the samples show an almost linear weight loss per temperature up to 75%, at 20°C. Figure 22 shows that weight losses at -5°C, 1°C or 10°C were quite comparable.
The dry matter percentage (DM%) of fresh beet leaves was about 12-15% (Figure 23). The samples measured later in storage showed higher DM% at -5 and at 20°C. At 20°C this can be partly explained by the loss of fresh weight (water). However it does not explain the result at -5°C. This weight loss

measured for leaves stored at -5°C may be underestimated as the leaves were frozen and weighed within one hour. It is possible water condensed on the leaves, which may interfere with the results.

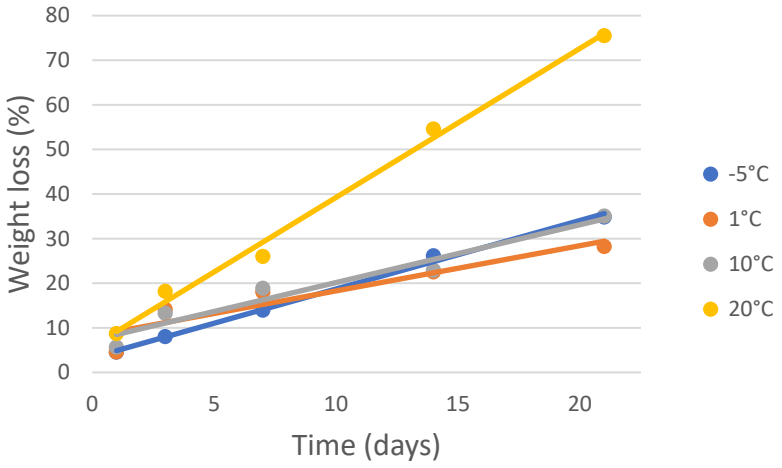

Figure 22: Percentage of fresh weight loss at various storage durations/temperatures. Lines are linear trendlines (n=3 samples of each 5 leaves)

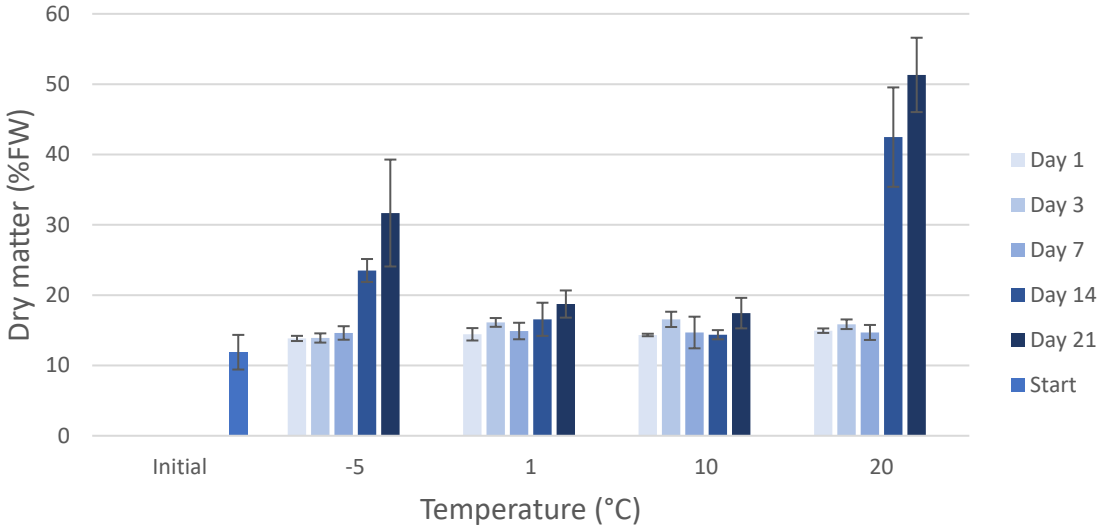

Figure 23: Percentage of dry matter at various storage durations/temperatures. Bars indicate 95% confidence interval (n=3 samples of each 5 leaf tips)

4.3.3 Protein content

Protein content was measured with BCA assay on extracts from leaf material and was expressed in mg BSA protein per g fresh weight (Figure 24-A) and in mg BSA protein per g dry matter (Figure 24-B). When expressed per gFW, an increase in leaf protein content was observed during storage at -5°C and 20°C after 14 days of storage. This will be partly related to the concentration effect caused by the changes in water loss. At the other storage temperatures leaf protein content showed little change during the storage. Leaf protein content expressed per gDW only showed a clear decline in the samples stored at 20°C. For the other storage temperature conditions, the total protein content remained more or less stable during the 21 days storage period.
The lower protein content observed for the storage at room temperature may be explained by the higher respiration rate (not measured) that can be expected at higher storage temperature and by the higher senescence rate (discolouration).

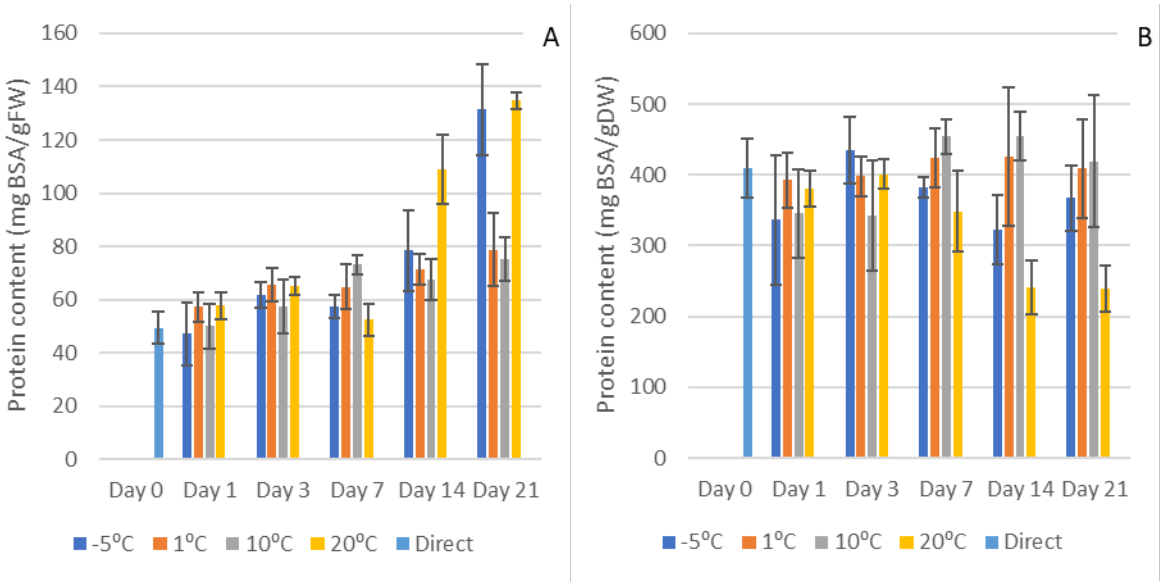

Figure 24: Protein content measured in sugar beet leaves after storage at -5°C, 1°C, 10°C and 20°C for a storage period of 0, 1, 3, 7, 14 and 21 days. Protein content is expressed in mg protein per g fresh weight (A) or per g dry matter (B). Bars indicate 95% confidence interval (n=3 samples containing leaf material from 5 tips)

The SDS gels (Figure 25) investigate the stability of the most predominant proteins during the storage treatments. The presence and abundance of Rubisco protein is characterised by two subunits located at 53 and 14kDa. In general the band at 53kDa is thicker and more intense in colour. The band at 14kDa is also present but at lower intensity. For this reason, remarks about the presence and stability of the Rubisco protein will be based on the band at 53kDa.

Regarding the effect of the storage temperature on the presence of Rubisco protein, only storage at 20°C for more than 14 days showed a negative effect on the rubisco content. There is also some aggregation of proteins visible at the injection area at the top layer of these slots indicating that a reaction between Rubisco protein and phenol may have occurred during the long storage (>14 days) at 20°C.

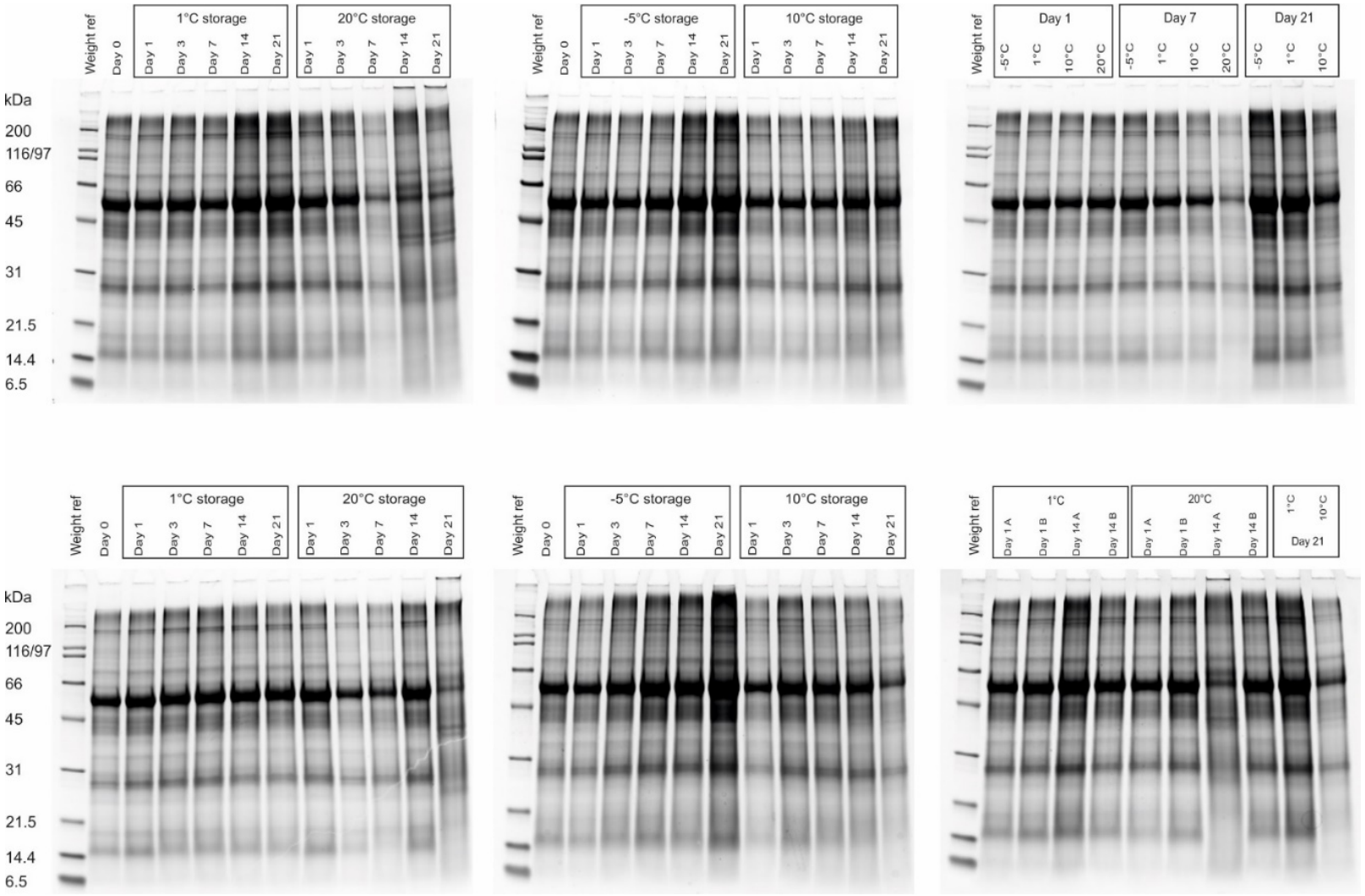

Figure 25: SDS page gels of proteins extracted from fresh leaves of sugar beet material stored for different periods at different temperatures. The upper figures show the protein content in the first replicate, the lower figures show the distribution of proteins in the second replicates; with exception of the lower right figure where samples stored at 1ºC and 20ºC for 1, 14 and 21 days were compared.

4.3.4 Total Phenolic Components

The total phenolic compounds (TPC) in the sugar beet leaves was measured with the Folin-Ciocalteu method and results are depicted in Figure 26. TPC can be found at a range of 150 µg GAE/g FW sugar beet leaf at harvest. When storing the leave at low temperature (-5 or 1°C), the TPC content remained stable during the complete storage period. At higher storage temperature, an increase in TPC per gram fresh weight was visible from day 7 and day 14 when leaves were stored at 20 and 10°C respectively (Figure 26-A). When expressing the TPC per gram dry weight (Figure 26-B), the results show for storage temperatures of 10°C and below, a relationship between higher storage temperature and higher TPC. However, for samples stored at 20°C for up to 2 weeks, it seems that the TPC decreased drastically. We hypothesize this may be a side effect of a too high dehydration process of the sugar beet leaves. Sugar beet leave stored at 20°C for 2 weeks showed more than 50% weight loss (Figure 22). This abuse weight loss may have resulted to severe tissue damages such as cell membrane breakdown for instance. This structure breakdown may have inhibited the total phenol production or allowed reaction with other plant tissue material (protein for instance). The leaves stored at 10°C did not show this abuse weight loss. Here the TPC at 10°C per gDW increased significantly between day 1 and 7. However, at day 14-21 the TPC per gDW remained stable.

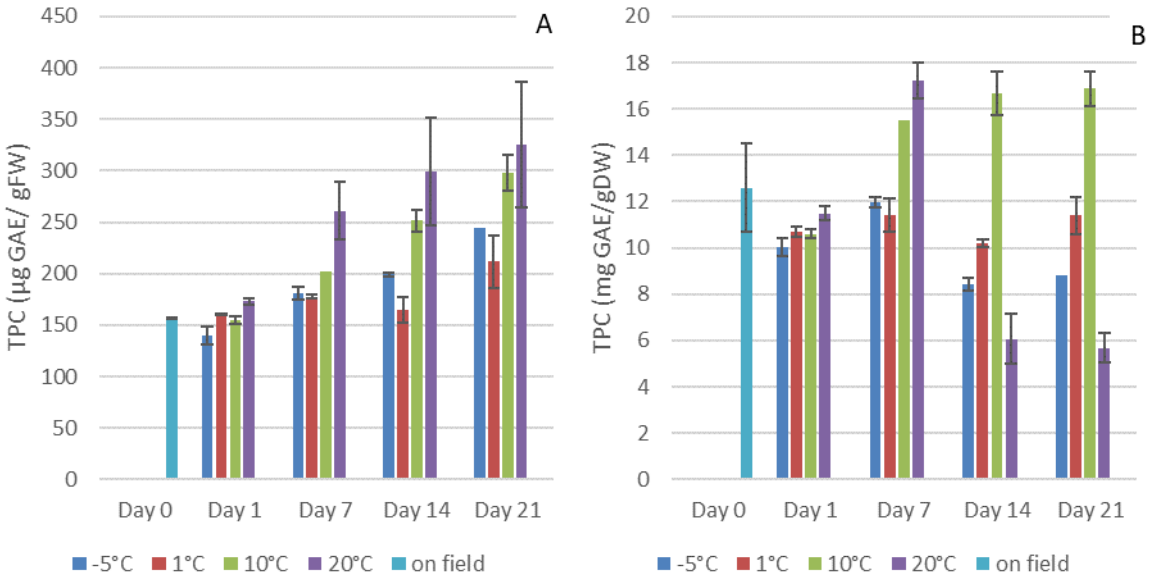

Figure 26: Total phenolic compounds (TPC) measured in sugar beet leaves after storage at -5°C, 1°C, 10°C and 20°C for a storage period of 0, 1, 7, 14 and 21 days. TPC is expressed in µg Gallic acid equivalent per g fresh weight (left figure: A) and in mg Gallic acid equivalent per g dry weight (right figure: B). Bars indicate standard error (n=2).

4.4 Conclusion & Discussion

Concerning the protein content of the sugar beet leaves as measured with both BCA method and the SDS-PAGE gel, the storage temperature and duration seem to have a limited effect within the first week; but both methods show a decrease in protein content after 2 and 3 weeks of storage at 20ºC. It also seems that the protein structure is affected by the storage conditions. The two rubisco subunits disappear on the gel after 3 weeks of storage at room temperature. Regarding these results, the protein in the sugar beet leaves seems to be stable for a period up to 2 weeks, independent of the storage temperature. From the present study, we can conclude that the protein remains stable for a relatively long period, when sugar beet leaves are stored intact. Storing at lower temperatures may increase this period.

Measurement of total phenolic component in the samples indicates that there is an increase in levels starting from 7 days storage at 10ºC and 20ºC. It is known that polyphenols may form complexes with proteins leading to changes in the structural, functional and nutritional properties of both components (Ozdal et al., 2013). However, due to the effect of abuse dehydration on the samples stored at 20ºC, it is not clear whether the aggregation of proteins in the top of the gels, after 14 days 20ºC, can be explained by only a reaction with phenols.

Yildiz et al. (2007) measure TPC in sugar beet shoot when grown on sugar enriched media. There was a positive correlation between the sugar content in the medium and the TPC in the sugar beet leaf material. The high TPC content measured on samples harvested directly in the field may be explained by the high sugar content stored in the beet root. It could be interesting to investigate the phenol content in the sugar beet leaves during the complete harvesting season. The sugar stoked inside the beet root may affect the phenol content in the sugar beet leaves and in a second step affects the stability of rubisco during post-harvest storage.

The results seem slightly contradictory with observations in earlier protein extraction experiments (internal discussion). It was reported that the protein in sugar beet leaves is unstable and should be extracted after harvest without any delay to realize high extraction efficiency. Our results show that the reason to do this is not because of fast degradation of proteins in the intact leaves. It is however not clear if the lower protein yield observed in these studies upon delayed extractions, is correlated to lower volume of extracted juice or lower protein levels.

5 Effects of dehydration/loss of turgor of sugar beet leaves on protein content and quality

5.1 Introduction

Another experiment was done with sugar beet leaves to determine the effect of dehydration on protein content and quality. From a physiological point of view, we don't expect a direct decrease in proteins of protein stability when dehydration takes place. However, because of the experiences in earlier studies (internal communication) of low extractions of Rubisco when extractions were not performed immediately, we would like to see whether this is correct. The following hypothesis was formulated:

- When a leaf is dehydrated (and turgor inside the cell is getting lower), the protein content and stability will not be affected.

Protein retention and stability was investigated for several turgor pressures. A scale of turgor pressures was obtained by storing sugar beet leaves at 20°C and 60% relative humidity or inside a box in which the relative humidity was kept near to 100%. The evaluation time was adjusted according to the water loss degree/turgor pressure reached during the storage period.

5.2 Set-up

Sugar beet was grown at Unifarm (Wageningen, the Netherlands, parcel lot HH2, HH3 and HH4) during 2019. At October 10th sugar beet leaves with the crown were harvested using a sharp knife. In this way the leaves were still connected and prevented from drying out. Liquid nitrogen was also transported to the field in order to sample sugar beet leaf just after harvest. The sugar beet leaves were transported by bike to the lab (± 10 minutes) and left outdoor until start of sampling. Prior to taking samples the crowns were put in a centrifuge, to remove the water from the leaves. Five leaves including stem of five different crowns were taken per sample, only leaves from the middle of the plant were taken, avoiding the older and younger leaves. The leaves were weighed and placed on perforated aluminium support (2 and 3 leaves per support, using 2 supports per sample). The supports were placed either at a table (60% RH, 20°C), or in a conditioned Perspex box, with 100% RH, 20°C (Figure 27). Samples for protein analysis and dry matter determination were taken after different time periods, targeting 1, 5, 10, 20 and 25% dehydration for the 60% RH samples as shown in Table 1. This was done in a similar way as described in 4.2. Every time period the remaining samples were weighed to track the water loss.
For protein extraction and analysis the same methods were followed as described in 4.2.3-4.2.5.

Table 1: Sample setup sugar beet turgor experiment

Sample	Time passed	60% RH	100% RH
WL0	0	X	
WL1	± 30 min	X	
WL5	± 2 h	X	
WL10	± 5 h	X	X
WL20	± 22 h	X	X
WL25	± 27 h	X	

Figure 27: Sampling the sugar beet. From left to right the crown with the leaves, sampled leaves on an aluminium support, sampled leaves on an aluminium support, inside the Perspex box.

5.3 Results

5.3.1 Weight loss and dry matter development

At 60% RH 20°C sugar beet leaves loose more weight during the first hours (see Figure 28). In these first hours the leaves might have lost weight due to evaporation of some remaining condensed water on the leaf caused by harvesting at slightly lower temperature and high humidity. The stabilization of weight loss may also be related to closing of the stomata, induced by the developing water stress. After 6h the weight loss seems to become linear in time. The weight loss in the hotbox (100% RH) is clearly less than at 60% RH. Figure 29 shows that the dry matter % increases over the time when stored at 60% RH and remains similar at 100% RH. The increased %DM at 60% RH is a result of the water loss (Figure 30).

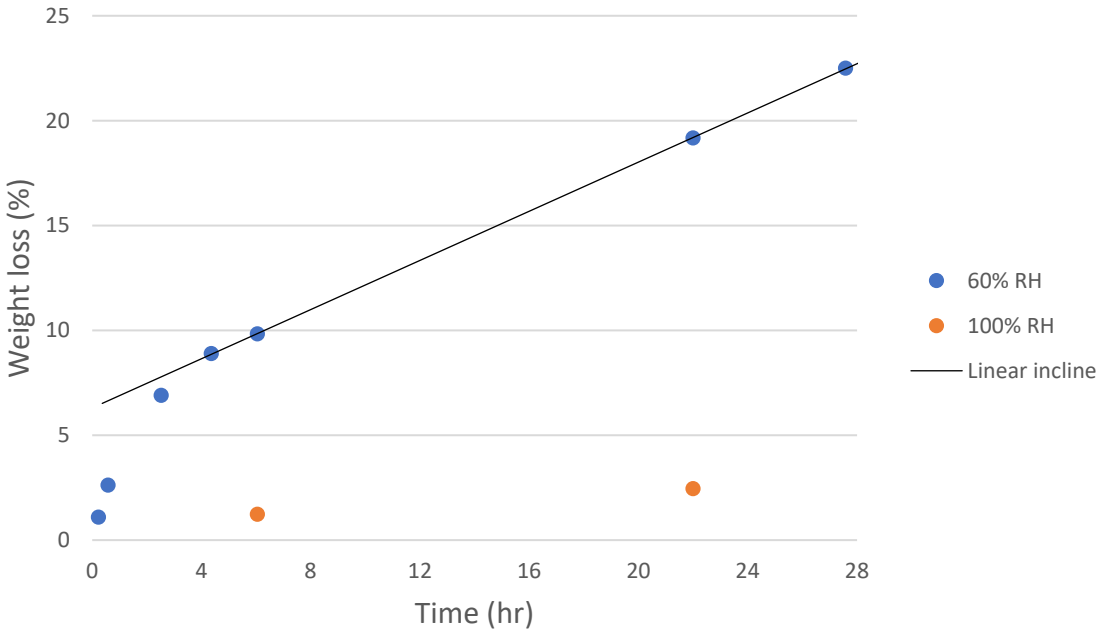

Figure 28 Percentage of fresh weight loss at various storage durations and relative humidity (RH) during the turgor experiment. After 4 hr, weight loss at 60% RH shows a linear incline (n=2, mixed sample of 2 or 3 leaves)

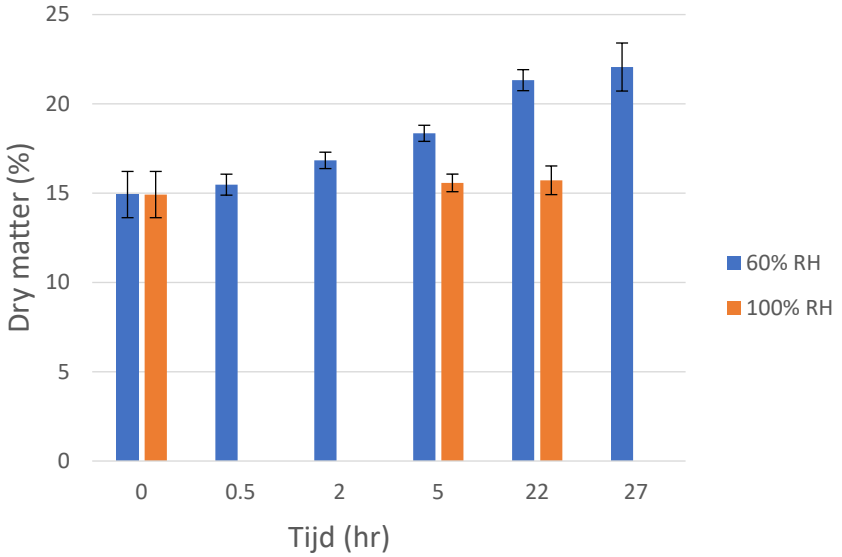

Figure 29: Percentage of dry matter at various storage durations and relative humidity (RH) during the turgor experiment. Bars indicate 95% confidence interval (n=2, mixed samples of 3 leaves)

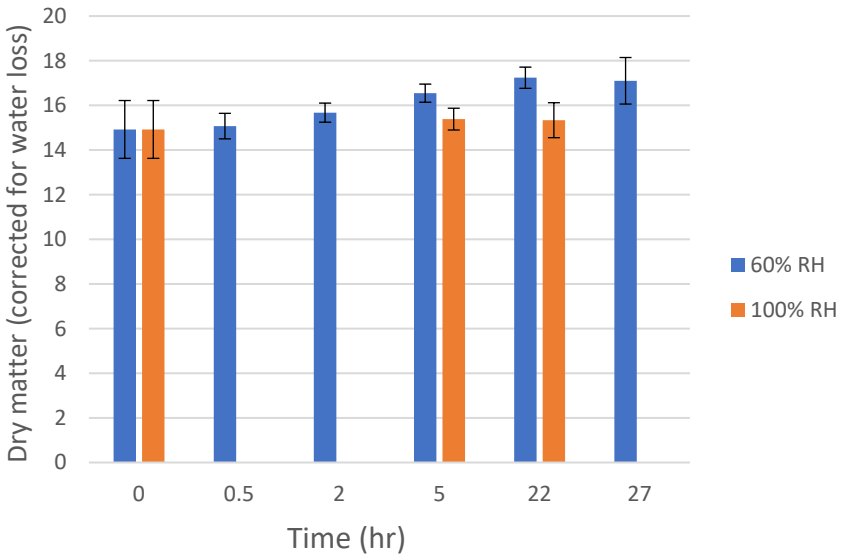

Figure 30: Percentage of dry matter corrected for water loss at various storage durations and relative humidity (RH) during the turgor experiment. Bars indicate 95% confidence interval (n=2, mixed samples of 3 leaves)

5.3.2 Protein content

Protein content as measured following BCA method and expressed in mg BSA/gFW shows a slight increase in samples stored at 60%RH (Figure 31). This increase is mainly due to concentration effect. Expressed per gDW, protein content showed a slightly decrease when leaves were subjected to more dehydration (Figure 32-B).

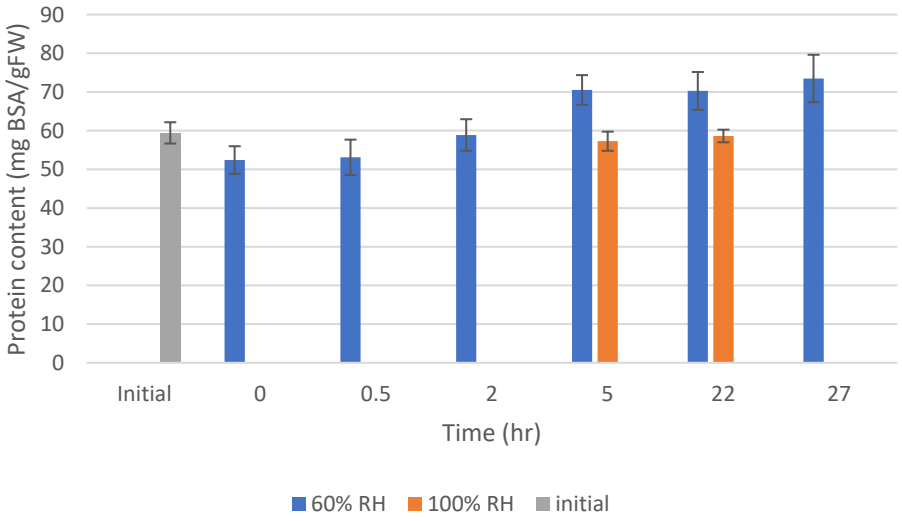

Figure 31: Protein content of sugar beet leave at various storage durations and relative humidity (60% or 100% RH) measured following BCA method and expressed in mg BSA/gFW. Error bars indicate 95% confidence interval (n=3).

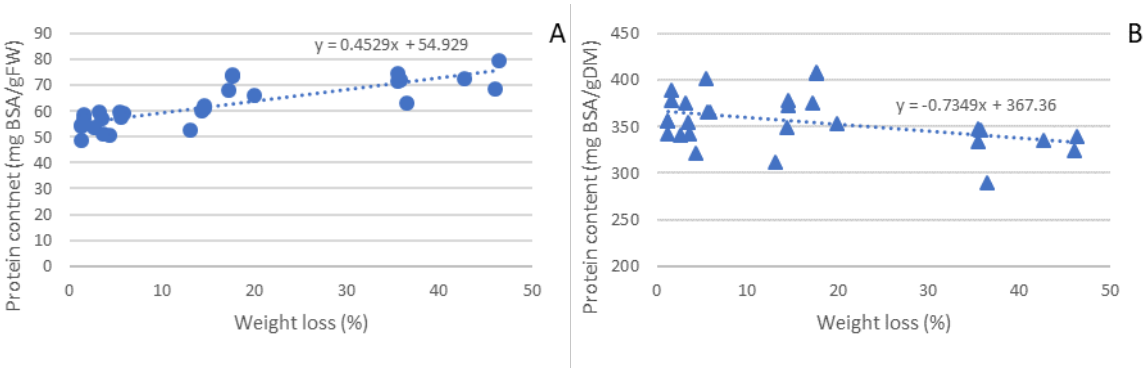

Figure 32: Protein content of sugar beet leave measured following BCA method and plotted against weight loss in percent. Figure A plots the protein content expressed in g fresh weight and figure B in g dry weight. On each figure trend line was added. Each dot represents one sample.

The results of the SDS-page gels (Figure 33) show that there is no clear change in protein content and composition, extracted from the leaves in dependence of time and desiccation. The samples from leaves which were left at 100% RH (marked with HB) are showing similar pattern to the starting samples.

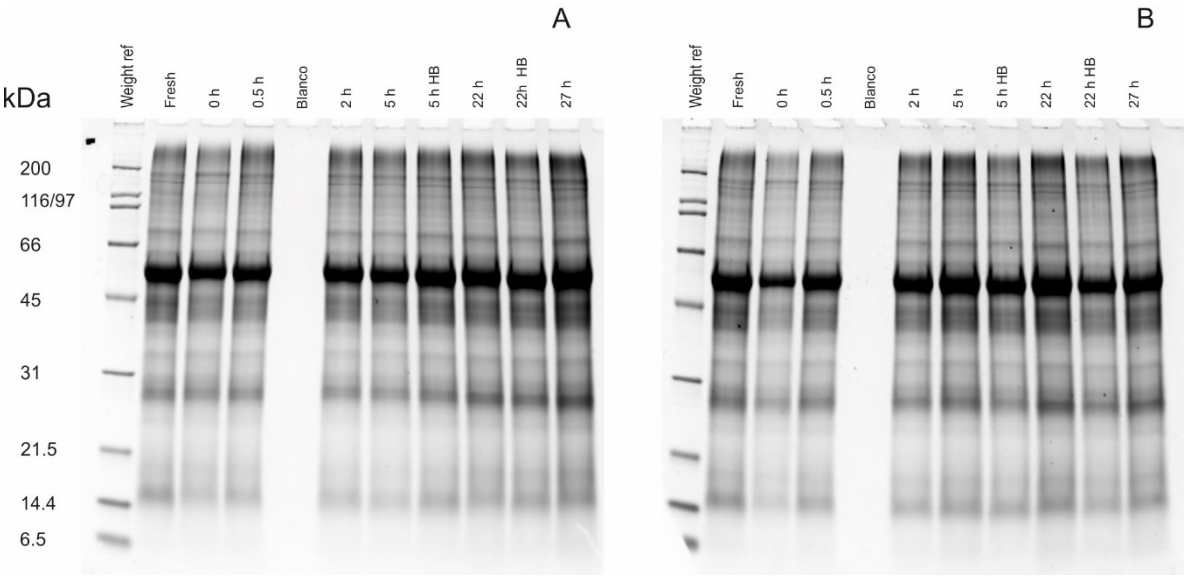

Figure 33: SDS page gels of proteins extracted from fresh sugar beet leaf material stored for different periods of time at 20°C and 60% relative humidity till reaching the targeted Water Loss (WL) percentage. Sample coded with HB are sugar beet leaves stored at 20°C and 100% relative humidity for the same period of time as the sample noted with time period. A and B gels are made of duplicate samples.

5.4 Conclusion & Discussion

Short term desiccation up to 20-25% in a room with 60% RH, 20°C, does not affect protein levels (corrected to dry weight) in sugar beet leaves. The protein composition seems also not affected. There is no clear indication on hydrolysis of proteins within this time frame and with respect to desiccation up to 25%.

6 Overall conclusions and discussion

This study shows a clear positive effect of cold temperatures during storage on protein retention and on Rubisco levels in Italian ryegrass and sugar beet leaves. Italian ryegrass (*Lolium multiflorum)* showed a better retention of protein content and quality when stored at low temperature -1 and 5°C, compared to 10 and 20°C. SDS-Page gels of extracts of these samples showed that after 3 days for all temperatures limited breakdown of proteins was seen, specifically looking at the large subunit of Rubisco. However, after 7 days at 10°C, and more particularly at 20°C, most protein is broken down. This protein degradation at higher temperatures was also reflected in a measured change of colour (ΔE) of the sample material. This colour change might be a useful candidate to monitor protein degradation during storage.

Sugar beet leaves also showed a stable protein content and profile at low temperatures (-1, 1, 5°C) compared to higher temperatures (10 and 20°C). In this case the decrease becomes clearly measurable after 14 days at 20°C and 21 days for 10°C in the BCA-analysis. On the gel also faster breakdown of Rubisco large subunit is seen at higher temperatures after 14 and 22 days.

These results also indicate a slower disappearance of proteins in sugar beet leaves than in grass samples. To optimize logistics for protein recovery from herbaceous biomass, it is recommended to study the effects of storage conditions on protein retention. It is also recommended to place temperature loggers in the product during storage, knowing that temperature is affecting the levels and composition.

It is known that polyphenols may form complexes with proteins which may affect functionality, structure and nutritional value. Therefore in one of the sugar beet leaf experiments, total phenolic components were determined in leaf samples after storage at different temperature conditions. Results showed that there is an increase in total phenolic compounds measured when stored 7 days and longer at 10° and 20°C. However, due to the effect of abuse dehydration of the samples stored at 20ºC (>50% of start weight), it is not clear whether the aggregation of proteins in the top of the gels of samples after 14 days storage at 20ºC, can be explained by only a reaction with phenols.

In earlier studies (Bruins, 2020) it was experienced that proteins in sugar beet leaves should be extracted directly after harvest without any delay, to prevent lower protein yields. Our results show that storage of the whole leaf at low temperature prevents fast degradation of proteins in the plant material. By consequence, immediate extraction at harvest is not required, when the material is kept at room temperature or colder. Also loss of turgor by dehydration of the leaves, is not directly influencing the proteins in the sugar beet leaves. Measurements in our experiment after short term desiccation (~ 1 day) of sugar beet leaves (up to 20-25% moisture loss, in a room with 60% RH at 20°C) did not seem to affect protein levels (based on dry weight%) and composition.

Possible reason for finding lower yields could be that cuts/wounds affect protein stability after harvest. It is also probable that the lower protein yield upon delayed extractions, is related to lower volumes of extracted juice. The loss of turgor might influence the pressing efficiency and therefore the amount and the extractability of proteins.

Further study is needed in order to investigate the practical application of low temperature post-harvest storage of herbaceous biomass for biorefinery purpose. For example, preventing big piles of non-temperature controlled biomass, by active or inactive cooling, may help to extent the storability of the raw material before protein extraction.

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