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

Part III (Sugar beet leaf harvest 2020)

CONFIDENTIAL

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Institute: Wageningen Food & Biobased Research

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Contents

Preface

This report is the third report 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. It describes the results of experiments executed in 2020, on the effect of storage conditions on protein stability of sugar beet leaves.
We would like to thank our colleagues from Unifarm (Gerard Derks and Johan van der Lippe) for support in collecting the sugar beet leaves from the field. Furthermore we would like to thank our colleagues again for interesting discussions and guiding us into the world of biorefinery and proteins: Stacy Pyett, Paul Bussmann, Edwin Keijsers, Peter Geerdink and Marieke Bruins.

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

Summary

This report is the follow-up of the exploration study concerning the potential impact of post-harvest technology on protein supply chains with special focus on the effects of post-harvest conditions on protein retention in sugar beet leaves. The research was performed independently by researchers from Wageningen Food & Biobased Research and funded by the Ministry of Agriculture, Nature and Food Safety through DFI- R&D budget, within the strategic WUR-KB theme of Healthy and Safe Food. The study has been divided into two main experiments: in a first part the effects of temperature and storage time on protein retention and on RuBisCo stability in sugar beet leaves were investigated. In a second experiment, the effect of big volume storage on temperature and RuBisCo retention was examined.

In the first experiment, sugar beet leaves were stored at 1, 20, 30 and 45 °C and sampled after 0, 6, 24, 30, 48, 72 and 168 h. The protein content, measured absolutely with both BCA method and relatively on protein gel, was stable during the first part of the storage, i.e. the latent phase. Depending to storage temperature, a decline in total protein content and in RuBisCo stability was observed. Based on these results, it was concluded that sugar beet leave can be stored up to 168 hours at 1 °C without major effects in RuBisCo degradation. When sugar beet leaves were stored at 20 °C, loss of RuBisCo was initiated after 72 h of storage, 48 h at 30 °C and between 24 and 30 h when leaves were subjected to a temperature of 45 °C.

In the second experiment, the effects of storing whole sugar beet leaf material in a big box at two temperature conditions was investigated. Sugar beet leaves were piled up in a big box to a height of 120 cm and left for 68 hours at 5 °C for the first box and at outside temperature for 24 hours followed by 44 hours at 20 °C for the second box. Each box was divided in 5 layers. Within each layer, temperature and gas contents were monitored over the complete storage period. At the end of this period and based on the temperature recordings, sugar beet leaves located at layers 30 and 120 cm at 5 °C and 0 cm, 30, 60, 90 and 120 cm at 20 °C were sampled for protein analysis. Protein extraction was done directly on both leaf material and juice extracted from 1 kg of raw leaf material. The first extraction method allowed comparison of protein content results to the first experiment and the second extraction method was comparable to standard extraction methods applied at industrial level. After 68 hours of storage (44 hours at 20 °C), a mild rise in temperature was observed at the centre of the box stored at 20 °C. The temperature recordings showed that the warmest spot was located at 60 cm depth (centre of the pile) and reached a maximum temperature of 31.9 °C for a short period of time (5.5 hours above 30 °C). Concerning the total protein content and RuBisCo stability analysis on samples extracted from the sugar beet leaf material, no significant difference between the two storage conditions (5 °C and 20 °C) and the different layers in the boxes was found. These results were consistent with the first experiment; the storage duration and the temperature rise observed in the boxes matched with the latent phase identified in the first experiment.

Based on the results, it is possible to conclude that sugar beet leaves can be stored for a period of 68 hours without major effects on the RuBisCo stability. Storage in a big box in a cold room set up at 5 °C helped to avoid the rise in temperature. RuBisCo was stable when the temperature inside the box remained under 30 °C for the majority of the storage period (62.5 hours).

Finally, predictive models based on dry matter and on non-destructive sensing technologies were investigated. The correlation between dry matter and protein content was judged poor and indicated that it is not possible to extrapolate the protein content of the sugar beet leaves on basis of their dry matter content. A model based on NIR-measurements was also built to non-destructively predict dry matter and total protein content of sugar beet leaves. A relatively good performance was achieved for the prediction of dry matter on basis of a 13-wavelength measurement. Unfortunately, the prediction of protein content was less sensitive when using a NIR measurement of 10 wavelengths. Further measurements are required to optimise this model improve its prediction score.

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 and animal feed is essential. Furthermore, to prevent negative effects of intensive agricultural production on land and water use, deforestation, and soil erosion; especially in vulnerable areas like the Amazon; it is important to stimulate the 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 for 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 yield, quality and functionality of protein-fractions or isolated proteins. There is sound indication 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 investigation into this issue has been done. This project explores the potential impact of post-harvest technology on protein supply chains. This Part III report presents results that are a continuation of the experiments with sugar beet leaves described in the Part I report (Paillart et al., 2020). Part II report describes results of post harvest conditions on proteins in yellow peas.

In the first part of the project, it was shown that low storage temperatures (< 10 °C) 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 20 °C. Also, it was observed that proteins in sugar beet leaves were stable for longer than in Italian ryegrass. This study shows that, at 20 °C and upon desiccation, protein degradation occurs over the course of days or weeks, rather than within a few hours. So the reason for finding lower protein yields at 20 °C is not due to fast degradation after harvest. However, higher temperatures in piles of biomass have been reported. Therefore, in two follow-up experiments, we have included higher temperatures and larger volumes of biomass, to better mimic conditions in practice. Also, not only the effect on protein content and quality was measured, but also the extractability of the proteins. The effect of storage with higher temperatures was investigated in the first experiment and is further referred to as experiment A in this document. The effect of storage with higher volume of biomass was studied in the second experiment and is further referred to as experiment B. The experimental design of these experiments has been described in Chapter 2 of this report, followed by the results and discussion in Chapter 3 and the conclusions in Chapter 4.

2 Material & Methods

2.1 Plant material

Sugar beet was grown at Unifarm (Wageningen, the Netherlands, parcel lot Born 02) in 2020. At October 19th and 20th of 2020, sugar beet leaves for experiment B and A, respectively, were manually harvested using sharp knives.
For experiment A, sugar beet leaves with crown were removed from the sugar beet root, stored in blue EPS crates and transported by car to the lab (± 10 minutes) (Figure 1-A). Since the leaves were dry, no centrifuge step was necessary to remove any free water. Triplicate samples were made per storage time point (0, 6, 24, 30, 48, 72 and 168 hours) and temperature (1, 20, 30 and 45 °C). Per sample, bunches of five leaves were made from a randomized selection of 10 crowns (Figure 1-C). Only leaves from the centre of the crown were taken, avoiding older and younger leaves. Leaves were bunched using a rubber band. Stems of leaves were re-cut in order to keep only 5 cm of the stem. Each bunch was coded, weighed (Figure 1-B) and photographed in a calibrated colour cabinet. Bunches were placed in trays with top lids, three per tray, and stored at 1, 20, 30 or 45 °C.
Per evaluation moment, one tray was removed from each storage room according to the planning summarized in Table 1.

Table 1 Temperature and duration storage

	Evaluation time (hours)						
Storage temperature	0 -Start	6	24	30	48	72	168
1°C			x		x	x	x
20°C	x *	x	x	x	x	x	x
30°C		x	x	x	x	x	x
45°C		x	x	x	x		

**: field temperature (around 20°C)*

On the evaluation day, pictures of the individual bunches were taken in the Smart Colour Inspector (SCI), see 2.2.2. Bunches were then individually weighed. Bunch of five sugar beet leaves was disassembled and leaf samples for protein and phenol analysis, and dry matter analysis were taken. Only the top 15 cm of the leaf tissue was selected for analysis. Each leave was divided in two sides when removing the midrib. One side was frozen in liquid nitrogen and stored at -80 °C for further protein and phenol analysis. The other side was put into a bag for dry matter analysis (see 2.2.4).

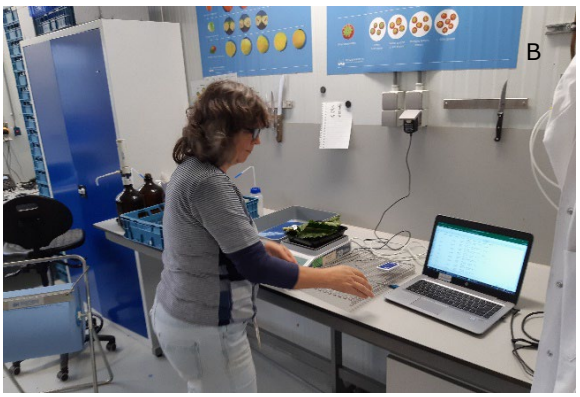


Figure 1 Impression of harvesting and preparation of samples sugar beet leaves.
(Source: Wageningen Food & Biobased Research)

2.2 Storage experiment high storage temperature

2.2.1 Storage conditions

Bunches of sugar beet leaves were kept inside plastic trays with transparent top lid. At the bottom of the tray a humidified tissue paper was placed to maintain a saturated moisture content in the tray headspace. The humidified tissue paper was covered with a metal grid to support the three bunches. Per evaluation day, one tray with the three bunches was prepared and stacked on top of each other (Figure 1-D).

In total four piles of crates were made and stored in four independent cooling rooms. The temperature of each cooling room was set up at 1, 20, 30 and 45 °C.

2.2.2 Colour images and analysis

Colour images of the sugar beet leave samples (3 replica's) were taken by laying each bunch of 5 leaves directly at the bottom of the cabinet on a white background. The cabinet was mounted with LED arrays on 5 sides (4038 K), designed by WFBR and built by IPSS Engineering (both Wageningen, Nederland). The cabinet was equipped with a RGB camera (MAKO G-192C POE, Allied Vision Technologies GmbH, Stadtroda, D) which took images from above using standardized settings with Colour Cabinet Software (WFBR, Wageningen , V1.6, 23-01-2019). Prior to each measuring session the system was calibrated using a white background (Forex® PVC sheet white 6mm) and a 24 squared colour cart (Colour checker classic, X-rite Europe GmbH, Regensdorf, S). Based on this calibration, the RGB images were transposed to the official L*a*b* (D50) values of the Macbeth ColourChecker (Pascale, 2006).

2.2.3 Near-infrared reflectance measurements and data modelling

Sugar beet leaves were analysed non-destructively with the visible and near-infrared spectroscopy using a portable spectrometer. The spectrometer was a Lab spec spectrometer from ASD (Malvern Panalytical, UK). The spectrometer recorded the data in the spectral range of 350-2500 nm with a spectral sampling of 1 nm. Special leaf clip, provided by the spectrometer manufacturer, was used to clamp the leaf during the NIR measurement. The sugar beet leaves were measured on two spots, on

the right and on the left side of the main vein as the reference measurements of dry matter and protein content were measured on two separate parts of the leaves. White reference measurements were also taken using a spectralon white reference and the reflectance was estimated as Eq 1:

$$Reflectance = \frac{I-D}{W-D} \quad [\%] \quad \text{Eq 1}$$

Where, I is the radiance, D is the dark reference, and W is the white reference.

The near-infrared spectra were used to calibrate the prediction of the protein and dry matter contents. Such calibration was performed to assess the capability of non-destructive measurements to replace the destructive analyses of protein and dry matter and to support real-time chemical properties prediction. In this study, two separate models were developed corresponding to protein and dry matter content. The modelling was performed using the variable combination population analysis (VCPA). VCPA was performed to not only develop the calibration but on other hand also to identify the key wavelengths of interest that contributed most to the prediction of properties. VCPA was a two-step procedure. First, an exponentially decreasing function (EDF) was employed to determine the number of variables to keep and continuously shrink the variable space. Second, in each EDF run, a binary matrix sampling (BMS) strategy that gave each variable the same chance to be selected and generates different variable combinations was used to produce a population of subsets to construct a population of sub-models. Then, model population analysis (MPA) was employed to find the variable subsets with the lowest root mean squares error of cross validation (RMSECV). The frequency of each variable appearing in the best 10% of sub-models was computed. The variables with highest frequency were the most important and vice versa. All data analysis were performed in MATLAB 2018b, USA.

2.2.4 Weight loss and dry matter content

For the weight loss determination, each individual bunch was weighed on a two digits balance (Toledo) and water loss occurring during the storage was calculated according to Eq 2 where W_n is the weight of the bunch after storage and W_0 is the initial weight of the bunch. Weight loss is expressed in % of weight loss**.**

$$WL = \frac{(W_n - W_0)}{W_0} \times 100 \quad [\%] \quad \text{Eq 2}$$

For the dry matter measurement, samples were taken as described in 2.1. A bag was first weighed empty (W_{empty}) and with the fresh material (W_{fresh}) with an analytical balance (3 digits). Bags were then dried in the oven at 80 °C for at least 48 hours. Bags with dry material were weighed (W_{dry}) at the end of the drying period. The dry matter content was calculated according to Eq 3 and expressed in percent.

$$DM = \frac{(W_{dry} - W_{empty})}{(W_{fresh} - W_{empty})} \times 100 \quad [\%] \quad \text{Eq 3}$$

2.2.5 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 protein, 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.

2.2.6 Protein gel (SDS-Page)

Changes in protein composition were examined with Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The stability of the RuBisCo protein was assessed based on the band from its large subunit as 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 with a saline solution (0.9 % NaCl) on basis of total protein content, measured with the BCA method to 2 mg protein / mL. For SDS-PAGE analysis the extracts were further 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 10 min at 95 °C in a mixing heating block and then centrifuged at 12000 g for 5 min. 7.5 µg sample protein was loaded on each slot of 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 analysed with the imaging system Chemo Doc touch (Bio-Rad) and Image Lab Software 6.1.

2.2.7 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 (see 2.2.5) 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 562 nm was measured with a spectrophotometer (Versomax). The Bovine Serum Albumin (BSA) calibration curve was used to calculate the protein content in µg BSA / mL. Results were expressed in mg (BSA) protein/g fresh weight and mg BSA protein/g dry weight.

2.2.8 Total Phenol analysis

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. 250 mg of frozen material was weighed into 2 mL costa tube. The extraction consisted of adding 1 ml of cold pure methanol to the leave material and one 5 mm tungsten carbide bead (Qiagen). The tube was shaken in 2 successive steps of 2 minutes at 30 Hz using a MM301 Vibration Mill (Retsch). Tubes were then gently shaken on ice for 1 hour at 250 rpm. Tubes were vortexed and centrifugated for 10 min at 15500 g and 4 ºC. Supernatant was then collected and filtered with 0.45 µm filter (Minisart Syringe filter from Sartorius, Goettingen, D). 100 µl of extracted sample was diluted into 6 mL demi water and 0.5 mL FC reagent. The solution was left for 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 765 nm was measured after incubation period with a spectrophotometer UV-3100PC (VWR, Radnor, PA).

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

2.3 Storage experiment effect high volume storage

In this storage experiment, we aim to identify the effect of storage under high volume on the RuBisCo stability and the extractability of the juice.

2.3.1 Storage conditions

Sugar beet leaves with stem were manually harvested in the second half of October 2020 at one of the WUR fields nearby the WFBR facility. Leaves were directly piled in two big wood boxes and transported to the WFBR facility for storage. No extra mechanical action was taken to compact the leaves in the box. Around 250 to 300 kg of sugar beet leaves were stored in each box. Boxes (L x B x H: 120 x 100 x 125 cm) were made of four multiplex wood panels to form the box side (Figure 3-B) and one grid at the box bottom which was covered with plastic foil.

In order to monitor the temperature within the bulk of the sugar beet leaves, the box was divided into five layers (Figure 2). At each layer, a plastic tube and two temperature loggers (Logtag) were placed. An extra temperature sensor (Escort data logger) was added on the layer located in the middle of the box (60 cm from the top) in order to record live temperatures (Figure 3-A). The plastic tube extended to outside of the box and connected to a pump (GilAir Plus, Sensidyne, St Petersburg, FL) and was used to sample air to determine oxygen and carbon dioxide concentrations. Oxygen and carbon dioxide concentrations at each level were measured daily using a Checkpoint 2 (Dansensor A/S, Ringsted, DK). Prior to each measurement, a pump was connected to the plastic tube of the respective level and switched on. Oxygen and carbon dioxide concentrations of this extracted air were measured using the Checkpoint 2.

Depending on the storage temperature, boxes were covered with plastic trays or with LPDE plastic foil for storage at 5 °C or outside followed by 20 °C, respectively. A few holes were applied in the plastic foil to ensure a sufficient O_2 and CO_2 exchange at ambient conditions. For the storage at 5 °C the box was immediately stored into the cool room set at 5 °C (Figure 3-B). After arriving at WFBR facility, the second box was first stored outside the building for 24 hours followed by 44 hours at 20 °C to stimulate any natural overheating inside the box.

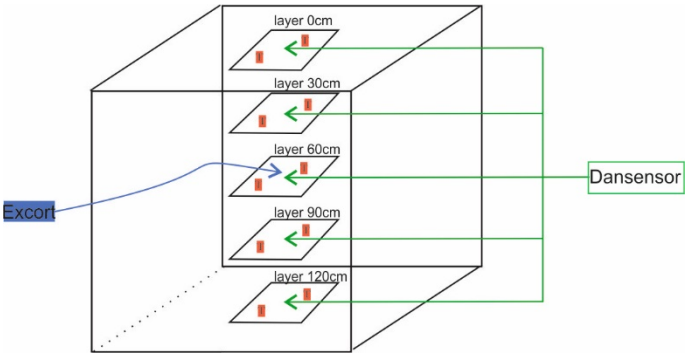


Figure 2 Schematic overview of the storage box divided in five layers from the top of the box. Positions of the Logtag temperature loggers are indicated by the orange square icons. Blue and green arrows indicate the inlet position of the Escort temperature logger and Dansensor instrument respectively.

Figure 3 ***Picture A shows the box filled half way with sugar beet leaves, the Logtag temperature sensors hanging at the predefined layers and Teflon tubing set at these layer for O2 and CO2 measurement. Picture B shows the filled box covered with plastic crates and stored in cooling room at 5 °C.***
(Source: Wageningen Food & Biobased Research)

2.3.2 Dry matter

Dry matter contents of the unprocessed leaf, the juice and the pulp were measured. Dry matter of the leaves was applied according to the method described in paragraph 2.2.4.

After juicing the leaves, dry matter content of the juice and of the squeezed pulp was measured in duplicate; 20 g of juice and 10 g of pulp were dried out in one oven at 80 °C for 48 hours. Dry matter content was calculated according to Eq 3.

2.3.3 Juice extraction and mass balance

For the juice extraction, complete sugar beet leaves with stem were used. 1 kg of leaves was pressed using the Angel slow juicer (Figure 4). In order to avoid binding of RuBisCo with phenol molecules released during juicing process, metabisulfite (MBS) was added to the juice to neutralize it. A fresh solution of 10 % MBS was prior prepared. At the start of the pressing, 10 ml of the 10 % MBS solution was added to the juice. Once the kilo of leaves was juiced, extra MBS was added to the juice to reach the required total MBS final content described in the patent of Jong de et al. (2014). Amount of MBS solution as well as the total volume of juice extracted were recorded for the mass balance.
The mass balance was expressed in percentage juice extracted and calculated according to Eq 4, where W_{leave} is the mass of leave before juicing and W_{Juice} is the mass of juice (MBS-free).

$$Mass\ balance = \frac{W_{Juice}}{W_{leave}} \times 100 \quad [\%] \quad \text{Eq 4}$$

Figure 4 ***Angel slow juicer instrument used for pressing the sugar beet leaves. Pulp was collected in the front container and the juice in the back container.***
(Source: Wageningen Food & Biobased Research)

2.3.4 Coagulation

In order to separate the cell tissue from the RuBisCo protein, a heat step called coagulation was applied within 2 hours after juicing. The pH of 80 mL sugar beet leave juice was first adjusted while

$CaCl_2$ was slowly added to the juice as described by Jong de et al. (2014). Two tubes (A & B) of 10.5 g juice with $CaCl_2$ were then incubated in a water bath, cooled down for 1 minute in ice water and centrifugated at room temperature.

The standard coagulation treatment and a short coagulation treatment were used to reveal differences in the samples when coagulation conditions are not optimal. The water bath and centrifugation specifications were:
Standard (A) 20 min 50 °C followed by 10 min centrifugation at 6500 rpm (rotor F14 14x50)
Short (B) 5 min 50 °C followed by 3 min centrifugation at 4000 rpm (rotor F14 14x50)
Supernatant was used for Bradford analysis (see 2.3.7), Protein gel migration (see 2.3.6), turbidity, chlorophyll and phenol content measurements (see 2.3.8) and size exclusion chromatography (see 2.3.9).

2.3.5 Protein extraction

Protein extraction was applied on the sugar beet leaves sampled on the bunch of 3 leaves and stored at -80 °C. Similar procedure that described in paragraph 2.2.5 was applied.

2.3.6 Protein gel (SDS-Page)

Samples extracted according to the protein extraction protocol (see 2.3.5) were compared to protein extracted from sugar beet juice combined with coagulation treatment. Both samples were first stored at -20 °C till analysis. Protein gel protocol was similar to the one described in paragraph 2.2.6. The volume of samples loaded on each gel-slot was adjusted to load similar amount of total soluble protein (Bradford analysis).

2.3.7 BCA and Bradford analysis

Total protein content of samples was measured both with BCA and Bradford analysis depending to the extraction protocols.

Samples obtained from the protein extraction applied on the sugar beet leave were, after thawing, analysed according to the BCA protocol as described in paragraph 2.2.7. Total protein content was expressed in mg (BSA) protein / g fresh weight and mg BSA protein / g dry weight.

Due to the addition of the MBS during the juicing process, only Bradford analysis could be applied on the juice samples. Soluble protein concentration in liquid samples was measured with the Bradford assay using Coomassie dye reagent (Pierce). The assay was performed in a microplate. 10 µL of sample was mixed with 300 µL of reagent. After 10 min the absorption was measured at 595 nm in a microplate reader (Versamax microplate reader). Bovine serum albumin (BSA) was used for calibration in concentrations of 0.2-1.0 mg / mL. Samples were diluted with 0.1 M NaCl to fit the calibration curve.

2.3.8 Turbidity, chlorophyll and phenol content

The turbidity, chlorophyll content and the phenol content of the supernatant obtained after coagulation were analysed using a spectrophotometer (Versomax).
The relative turbidity of the supernatant was analysed by adding 240 µl of milliQ water to 60 µl of supernatant. The absorbance of this dilution was then measured at 800 nm wavelength.
For the relative phenol content, supernatant was diluted 25 times in milliQ water before measuring absorbance at 340 nm wavelength.

For the relative chlorophyll content measurement, 900 µl cold acetone was added to 100 µl supernatant. After vortex, the sample was centrifuged for 5 minutes at 15000 x g
The absorbance of the supernatant was then analysed at 664 nm (in a quartz cuvette).

2.3.9 RuBisCo content measured with Size Exclusion Chromatography (SEC)

Size-exclusion chromatography (SEC) was performed on a Superdex 200 HR 10 / 10 column (GE Healthcare). The column was equilibrated with elution buffer (25 mM Tris / HCl at pH 8.0 with 0.2 M NaCl) at room temperature. Sample aliquots of 100 µL were injected and separated on a ÄKTA pure 25 system (GE Healthcare) at a flow rate of 0.4 mL / min. Absorbance was recorded at a wavelength of 280 nm. The system was calibrated with the following proteins (Amersham Pharmacia Biotech): thyroglobulin (660 kDa), ferritin (440 kDa), aldolase (158 kDa), albumin (67 kDa) and ribonuclease A (13.7 kDa).

The calculation of RuBisCo was performed by integration of the peak corresponding to the molecular weight of RuBisCo (550 kDa) using the extinction coefficient for RuBisCo at 280 nm ($A_{0.1\%=}$ 1.57).

2.4 Statistical analysis

Total phenol content in the first experiment, phenol absorbance and dry matter contents in the second experiment were analysed by via General Analysis of the Variance with an unbalanced treatment structure. Significant differences among treatments corresponded to Fisher Unprotected test at $p < 0.05$. All statistical analyses were carried out in Genstat 19 (VSN International Ltd, Hemel Hempstead, UK).

3 Results and discussion

3.1 Storage experiment high storage temperature

3.1.1 Storage conditions

The samples were stored for a period up to 168 h (7 days) in storage rooms in which temperature and relative humidity were controlled and monitored. Bunches of sugar beet leaves were stored in closed boxes with humidified tissue paper at the bottom. Due to poor airtight sealing of some boxes with the top lid, abuse dehydration has been observed on samples stored at 45 °C. These specific samples have been discarded from the results.
The temperature recording as well as the sample moments (orange arrows) are plotted on Figure 5. The targeted conditions were realized. The figure shows a drop in temperature between 24 and 30 h for the 45 ºC treatment. The temperature sensor was accidentally removed to the sampling location. The samples remained in the storage room at 45 ºC.

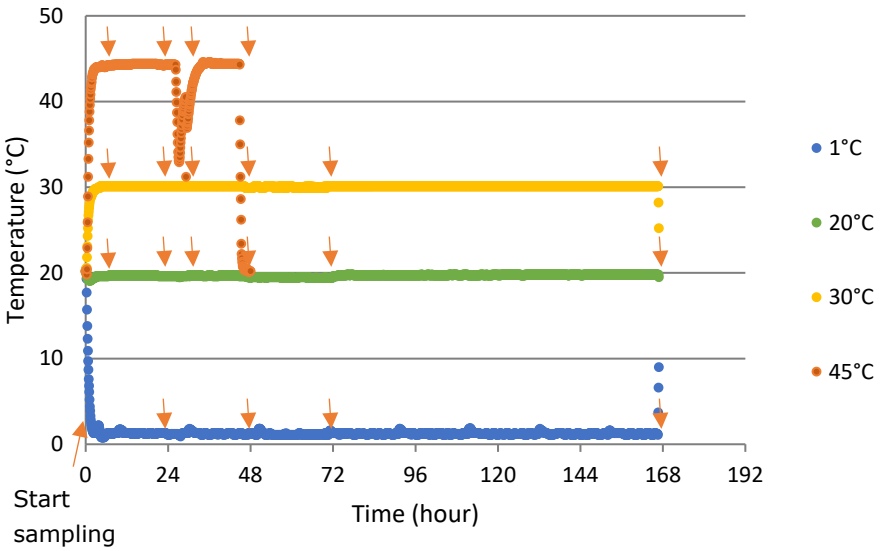


Figure 5 Temperature recordings during the 168 h storage period. Orange arrows indicate sample moment for each temperature treatments.

3.1.2 Colour

To provide a first impression of discoloration over time at different storage temperatures, pictures of the leaf-bunches were taken and placed into an overview matrix (Figure 6). Due to the removal of dehydrated leaves, discolouration linked to dehydration processes is not represented within the matrix. Samples stored at 20°C and 30°C for up to 168 hours showed brownish spots on the leaves due to unhealthy plant material.
Leaves remained green when stored for up to 168 hours at 1 °C, showed light yellowish discoloration after 168 h at 20 °C is yellowish, and brown discoloration after 168 h at 30 °C. Leaves stored 48 h at 45 °C showed darker colour.
L, a and b values of the leave samples were also calculated from the calibrated pictures (data not shown) but yielded no clear results due to inconsistencies in the colour due to shading and colour differences between the upper and lower sides of the leaves.

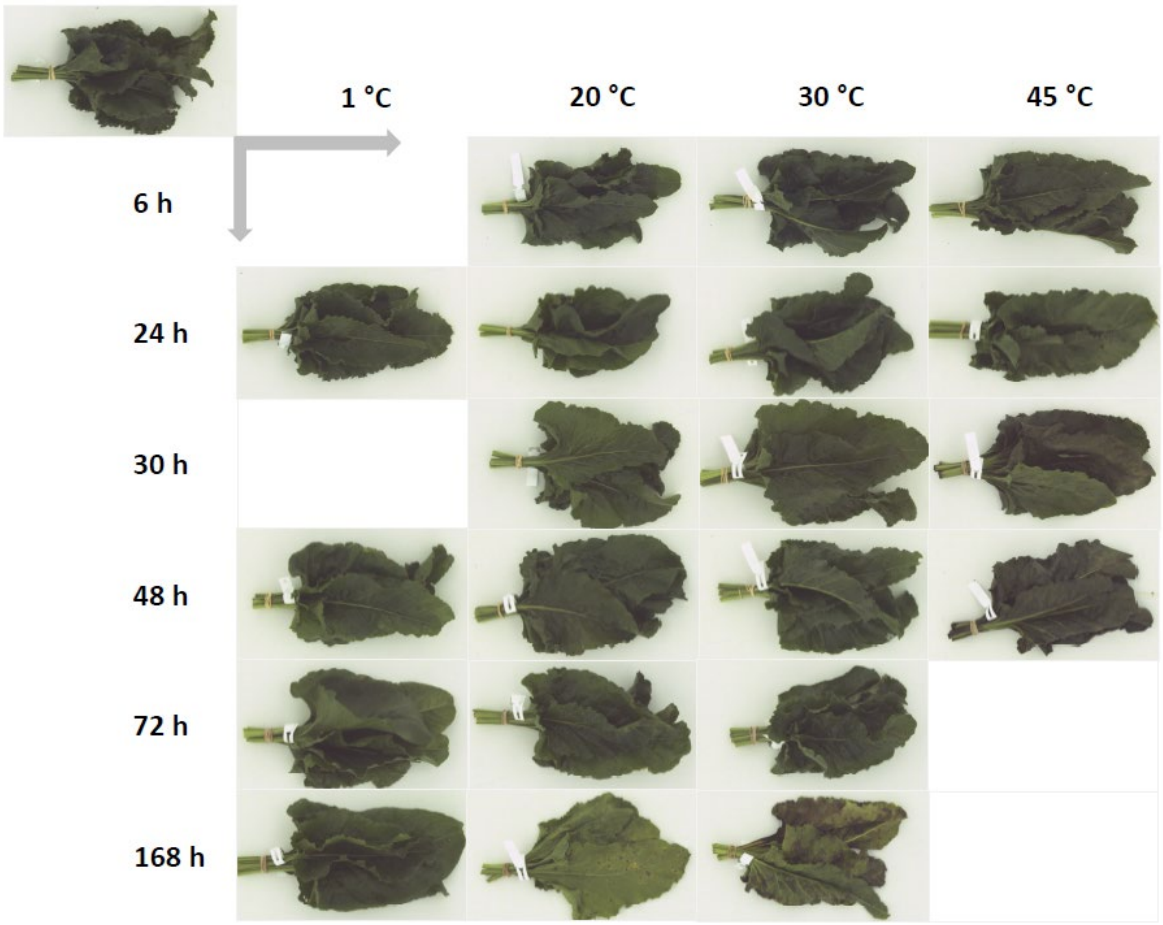


Figure 6 Matrix of calibrated pictures of sugar beet leaves samples after different storage periods (in hour) per row and different temperatures (in °C) per column. Picture of fresh sugar beet leaves at start is located on the top left.

3.1.3 Weight loss and dry matter

The weight loss and the dry matter content of the sugar beet leaves were followed during the storage. Figure 7 shows the relative weight loss at different storage temperatures and sampling times. The weight loss of samples taken on day 0 (start field) was per definition equal to 0 %.

Samples stored at 20 °C for 48 h and 168 h, as well those stored at 30 °C for 168 h showed very high weight loss values that were likely caused by not well closing lids. Furthermore, brownish spots were observed on samples stored for 168 h at 20 °C and 30 °C. This indicated unhealthy leaf material that may explain the higher weight loss observed in these samples.

When excluding these samples, a trend can be observed between the storage temperature, the storage duration and the weight loss severity. Higher storage temperatures did not only increase the weight loss of the leaves but also increased the respiration rate, which likely contributed to an increased dehydration.

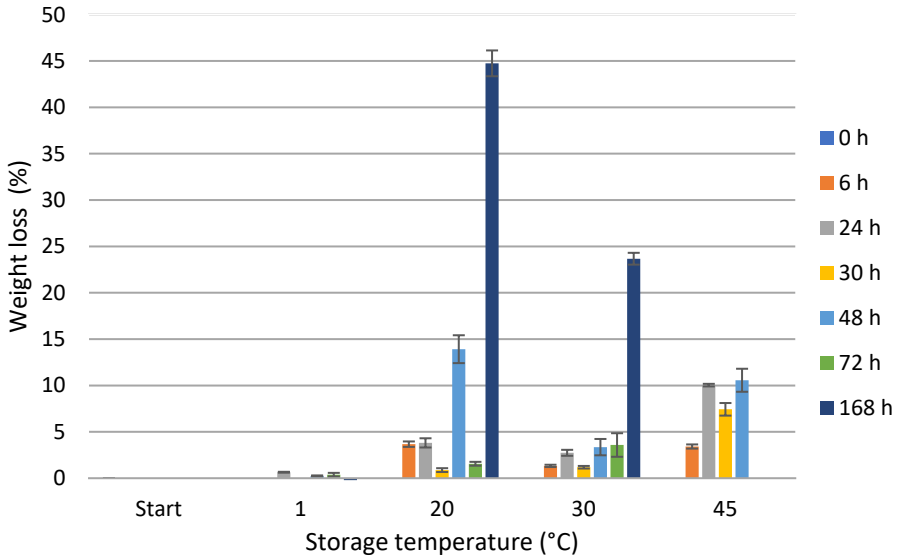


Figure 7 Percentage of fresh weight loss of sugar beet leaves stored at 1, 20, 30 and 45 °C for several periods. Each bar shows the average weight loss of three bunches of 5 leaves each (n = 3), error bar indicates the standard error.

The effect of the storage temperature and storage duration on the dry matter content is plotted in Figure 8. When discarding the higher dry matter content of samples stored at 20 °C and 30 °C for 168 h, no significant effect of the storage temperature and of the storage period was observed on the dry matter contents of the sugar beet leaves (Figure 8-A).

The higher dry matter content for these two samples should be considered as outliner since they were resulting to a measurement artefact. Dry matter was calculated according to Eq 3, using leaf weight at sampling moment as fresh weight. By consequent the dry matter results combined the dry matter content and the concentration effect due to weight loss occurring during the storage period. This effect was specifically visible for dry matter content of samples stored for 168 h at 20 and 30 °C and to a lesser extend for samples stored 48 h at 20 °C. The dry matter contents of these samples were significantly higher due to the artefact that the "fresh" materials were more dehydrated (Figure 7) than for other samples.

To remove this artefact, the dry matter was corrected with the weight loss occurring during the storage period (Figure 8-B). It seems that a correlation between the temperature and the storage period could be identified. Samples stored at 1 °C remained during the 7 d of storage with similar dry matter content over the time. However, for samples stored at higher storage temperatures, corrected dry matter content in general has tendance to decrease slightly over the time during the storage period.

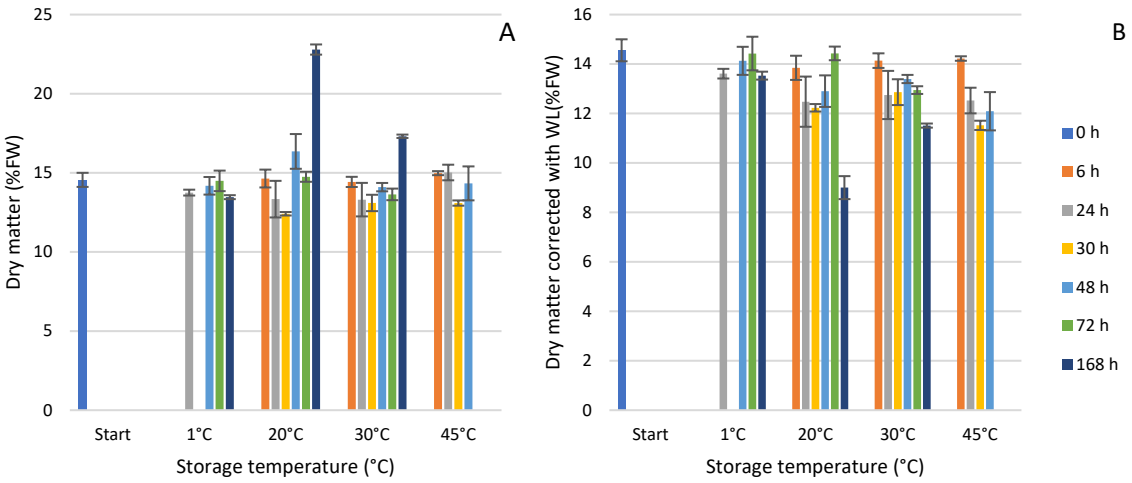


Figure 8 Percentage of dry matter (A) and dry matter corrected from weight loss (B) of sugar beet leaves stored at 1, 20, 30 and 45 °C for several periods. Each bar shows the average dry matter of three bunches of 5 leaves each (n = 3), error bar indicates the standard error.

3.1.4 BCA analysis

The total soluble protein content (TSP) present in the sugar beet leaves after storage was analysed on fresh (frozen) samples using BCA analysis. The TSP content is expressed per gram initial fresh weight sugar beet leaf (Figure 9-A) and per gram dry weight at the sampling moment (Figure 9-B).
During the first 24 hours of storage at 45 °C, the total protein content remained around 72 mg/g FW before decreasing to 27.7 mg/g FW after 48 h storage. Similar patterns with some delay and with lower extend were observed for the other storage temperatures. With exception of the samples stored for 72 h at 1°C, no decline of the total protein content was observed when sugar beet leaves were stored at 1°C. In general and irrespective of the storage temperature, it seemed that the total protein content started to decline only after a latent period that may match with the time needed for physiological processes to be initiated when plant materials were exposed to storage stress.

Results expressed in mg total protein per gram dry weight showed a similar pattern. However, these values differed from results reported in other studies. According to Tamayo Tenorio (2017), sugar beet leaves consist of 12% DM, which in turn is composed of 14.8 wt% protein, 2.9 wt% fat, 30.3 wt% total dietary fibre and 20 wt% ash on a dry weight basis. These values were obtained from whole leaves, including midribs and stems. When only extracting protein from leaf material, protein content increases to 21.6 wt% (Tamayo Tenorio (2017)). In the present study, only leaf material (from between the midribs) was used for the protein extraction and the dry matter content and up to 50% of the DW material consists of protein (Figure 9-B). This higher protein content may be partly explained by the type of leaf material used for the protein extraction and partly by the specific protein extraction buffer used to extract the protein out of the fresh material.

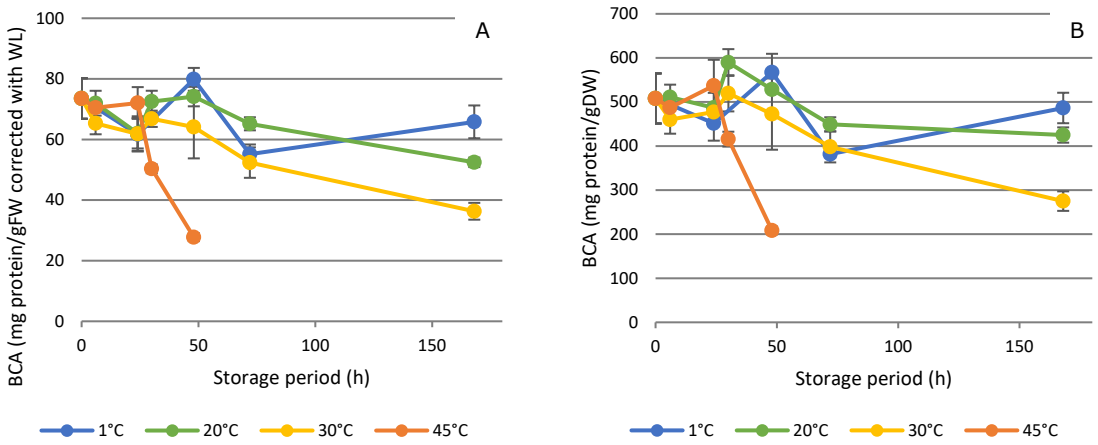


Figure 9 Protein content measured in sugar beet leaves stored at 1, 20, 30 and 45 °C for a period of 0, 6, 24, 30, 48, 72 and 168 h after harvest. Protein content is expressed in mg BSA protein per gram initial fresh weight sugar beet leave (A) and in mg BSA protein per gram dry weight (B). Bars indicates the standard error. (n = 3 samples containing leaf material from 5 tips).

3.1.5 Protein gel (SDS-Page)

Thanks to the migration of the protein through the SDS-Gel, it is possible to estimate the most abundant protein (RuBisCo) present in the samples. Using the protein concentrations determined by the BCA analysis , similar amount of protein were loaded on to the SDS-PAGE gels (+/- 10 %, standard BCA error assumption). The Rubsico band intensity correlates consequently to the amount of (RuBisCo) protein relative to the total soluble protein. This allows comparison of the relative amounts of RuBisCo between samples.

RuBisCo protein, when non-denatured, is made of 4 large sub-units of 53kDa and 4 small sub-units of 14kDa. In a previous experiment with sugar beet leaves protein extracts, following the same protein extraction protocol, the smaller sub-units did not appear on the SDS-Page gels (Paillart et al., 2020). For this reason, remarks about the effect of storage temperature on the RuBisCo protein stability have been based on the presence and intensity of only the large sub-unit (53kDa) band.

Figure 10 shows the effects of storage temperature over time on the relative amount of RuBisCo. When comparing the three replicates, similar patterns can be observed. This indicates that the reduced intensity of the large sub-unit band (53kDa) is triggered by the high storage temperature and the storage duration.

When sugar beet leaves are stored at 45 °C, high intensity of the large sub-unit band are visible for samples stored up to 30 h. For samples stored at 30 °C, large sub-unit band remains intense for storage till 30 h and slightly lightened from intensity when leaves are stored for 48 and 72 h and completely disappeared when storage was extended to 168 h. This indicates that the RuBisCo protein starts to degrade after 30 h storage at 30 °C.

For storage at 20 °C similar degradation pattern was observed with a slight delay in the lightening of the 53k Da band. This indicated that the storage at 20 °C does not inhibit the RuBisCo protein degradation but slowed it down significantly to allow a storage up to 72 h without major RuBisCo protein degradation. Sugar beet leaves stored at 1 °C showed the best RuBisCo retention over the complete duration of the storage period as the band intensities between the four storage time periods were similar.

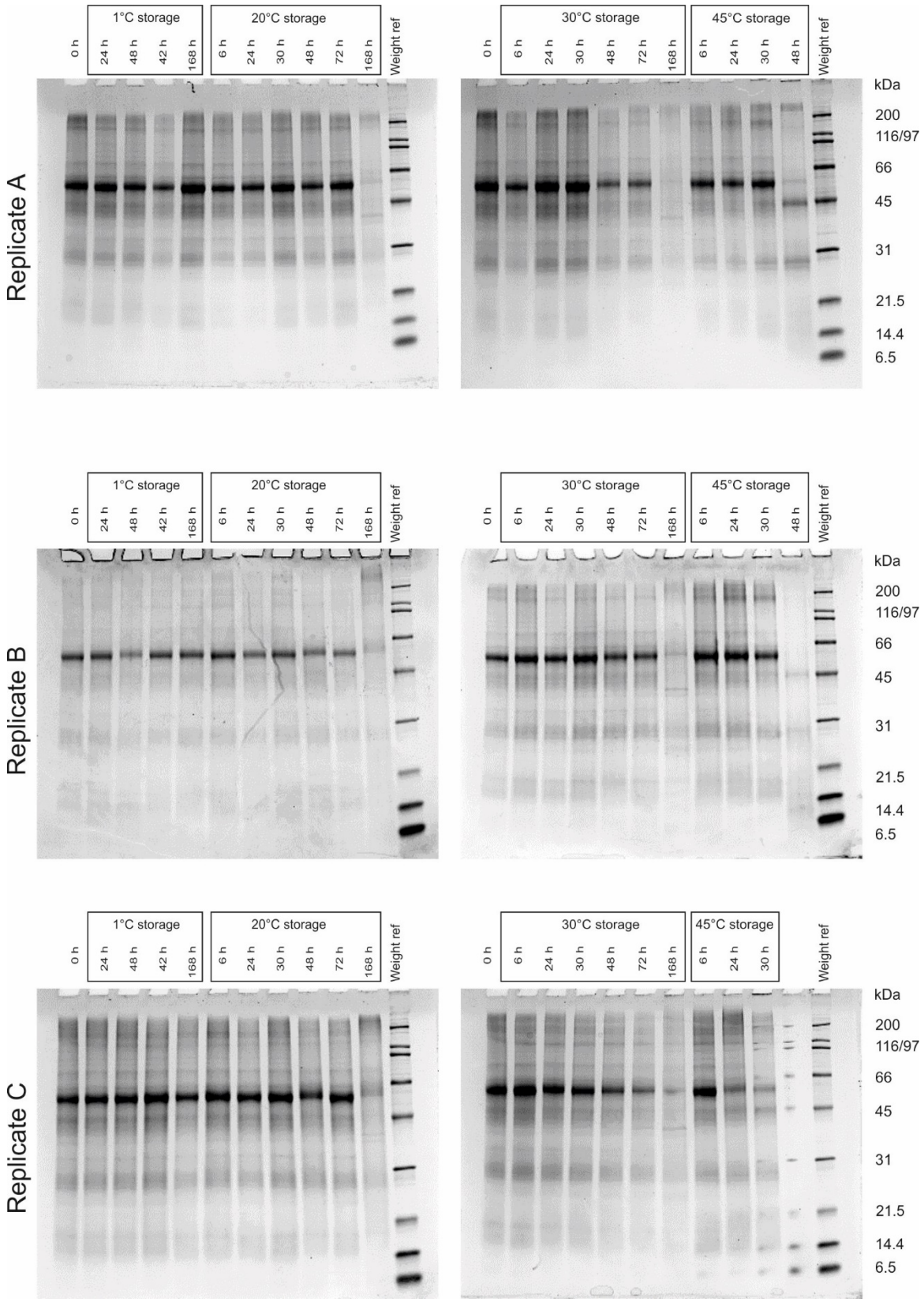


Figure 10 SDS-page gels of proteins extracted from fresh leaves of sugar beet leaves stored at 1, 20, 30 and 45 °C for 0, 6, 24, 30, 48, 72 and 168 h. The upper two figures show the protein content in the first replicate, the middle ones of the second replicate and the lower two figures of the third replicate. Slot on the right side of each gel shows the Low Molecular Weight (LMW) standard going from 6.5 to 200 kDa.

Figure 11 summarizes for one replicate the effect of four storage temperatures at different storage time. By comparing the band intensity of samples stored at different temperature, it is possible by storage time to identify the best storage conditions. When sugar beet leaves are stored for 30 h or

shorter, no effect of the storage temperature on the band intensity was observed. After 48 h storage, 30 °C storage showed a less intense band at 53 kDa and no band for sample stored at 45 °C was observed. RuBisCo protein were completely degraded between 72 and 168 h storage at 20 °C and 30 °C. When storing the sugar beet leaves for up to 168 h, only storage at 1 °C still showed high intensity band at 53 kDa.

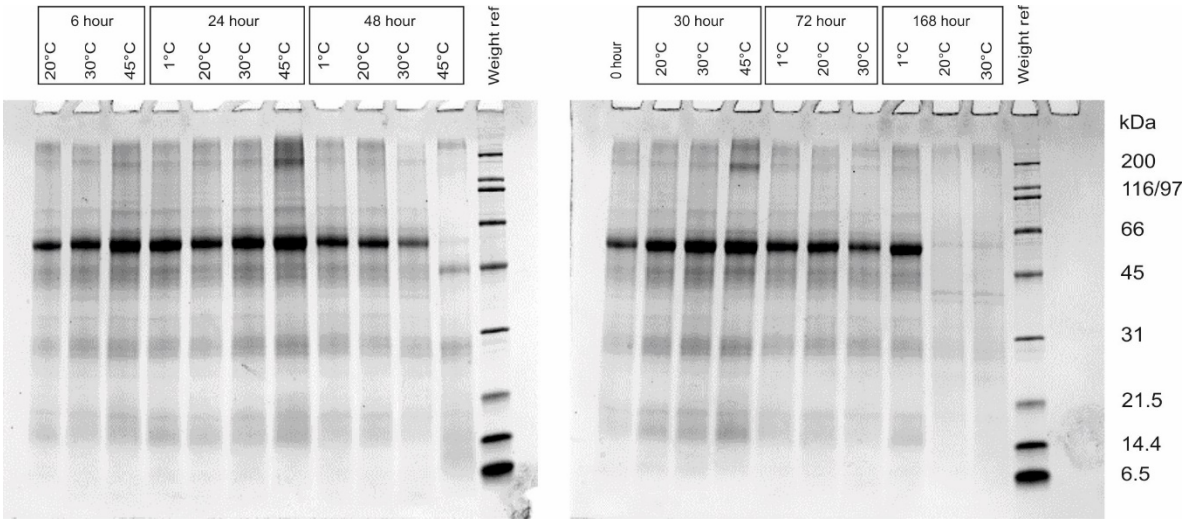


Figure 11 SDS-page gels of proteins extracted from fresh leaves of sugar beet leaves stored at 1, 20, 30 and 45 °C for 0, 6, 24, 30, 48, 72 and 168 h. These gels plots the effect of storage temperature at fixed evalaution moment. Slot on the left side of each gel shows the Low Molecular Weight (LMW) standard going from 6.5 to 200 kDa.

3.1.6 Total Phenol analysis

The Total Phenolic compounds (TPC) were analysed on the frozen samples according to the Folin-Ciocalteu method. Figure 12 summarizes the TPC content per gram dry matter and per gram fresh weight corrected with the weight loss.

Although the sugar beet leaves were stored intact, without any wounding, a slight increase of TPC can be observed after the first 24 h of storage at 45 °C, when leaves were stored at a lower temperature, milder and delayed increase of TPC was observed. Samples stored at 1 °C for up to 168 h did not show significant increase of TPC over the time.

For samples stored at the highest temperatures, a decrease of TPC was observed on the last evaluation day (after 48 h for storage at 45 °C and after 168 h for storage at 30 and 20 °C). Since phenolic compounds are produced by live cells and the leaves stored at 20, 30 and 45 °C were looking senescent at the end of their respective storage, the observed decrease in TPC content is possibly linked to leaf senescence. Leaf senescence is a common leaf response in cases of severe stress, conditions that may also trigger increases in the production of phenolic compounds, which would explain the sharp increase at 45 °C, followed by a decline as the leaf material succumbs to the stress. A similar thing is also visible at 30 and 20 °C, albeit at much lower rates.

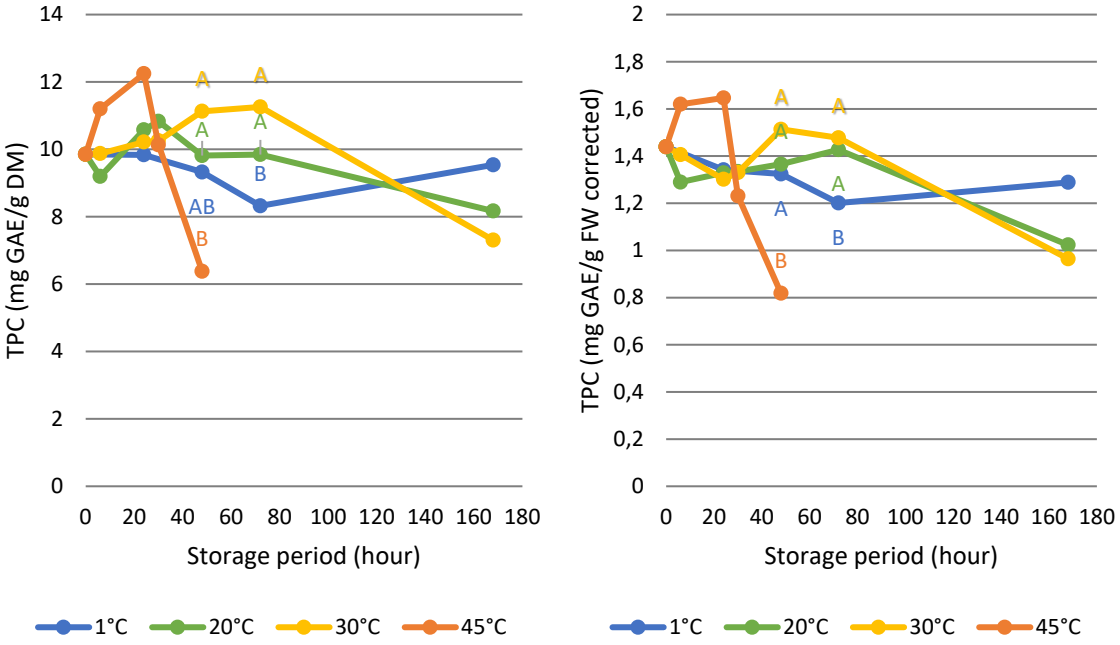


Figure 12 Total phenolic content (TPC) per gram dry matter (left figure) and per gram fresh weight corrected with weight loss (right figure) of samples sugar beet leave stored up to 7 days at four storage temperatures (1, 20, 30 and 45 °C). Significant difference between the storage temperature per evaluation moment are marked with letters according to Tukey test ($p < 0.05$) ($n = 3$).

3.1.7 Potential of non-destructive sensing for modelling protein and dry matter in sugar beet cuttings

This study involved developing a model for two different chemical properties i.e. dry matter and protein content. At first, a relation between the DM and protein content was explored and plotted in Figure 13. A slight negative correlation (~40 %) between the DM and protein content was noted. Such a negative correlation indicates that the samples with high dry matter attained lower protein content, however, the correlation seemed too low to conclude if it is really significant. In some fresh produce the dry matter is usually highly correlated with chemical components such as oil content and in such case the DM can be used as the proxy of such chemical components. However, in this study, due to a low correlation between DM and protein, it seems that prediction of DM as a proxy to asses protein content is not a feasible option.

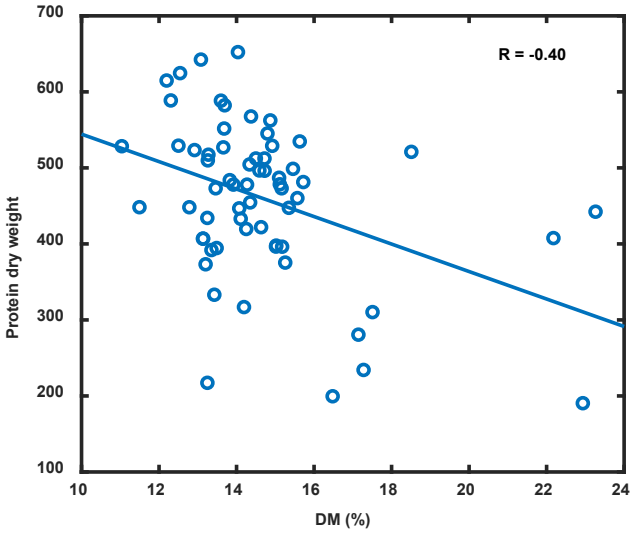


Figure 13 Dry matter vs protein content for sugar beet leaves.

Figure 14 and Figure 15 show the performance of NIR models for predicting DM and protein contents, respectively in fresh sugar beet leaves. Alongside the calibration results, the key wavelengths selected by the model are plotted. They are explained in Table 2.

A relatively good model for prediction of DM in fresh beet leaves was attained with an error of 0.608 % with as low as 13 key VISNIR wavelengths. The model performance for predicting protein content was slight poorer in terms of R^2. However, the result for protein shows that a good model can be foreseen with NIR modelling. A key point to note is that the prediction of protein content was possible with as low as only 10 key wavelengths. Summary of key wavelengths that can be used in future research for optimal model development can be found in Table 2.

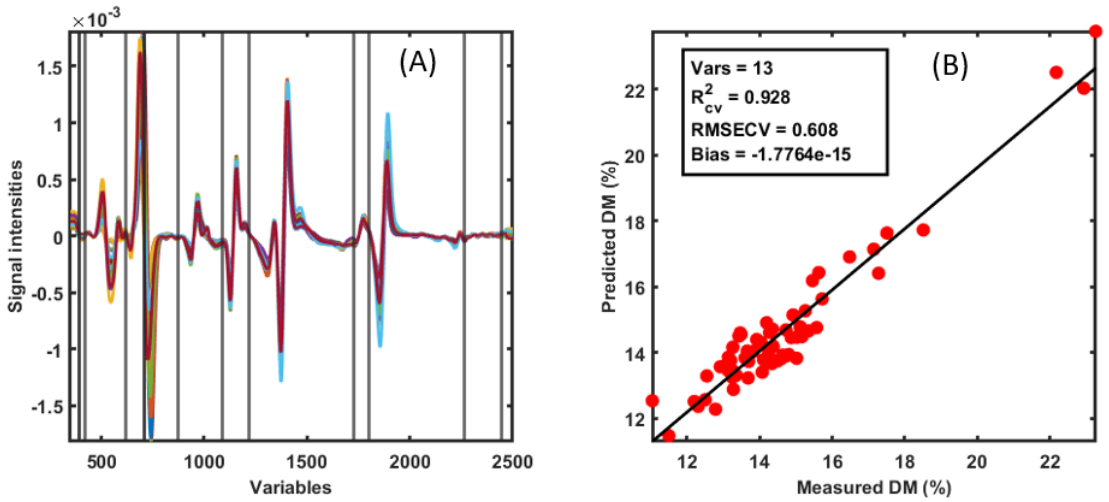


Figure 14 Summary of near-infrared prediction models for dry matter content. (A) Key wavelengths of interest, and (B) measured vs predicted DM (%).

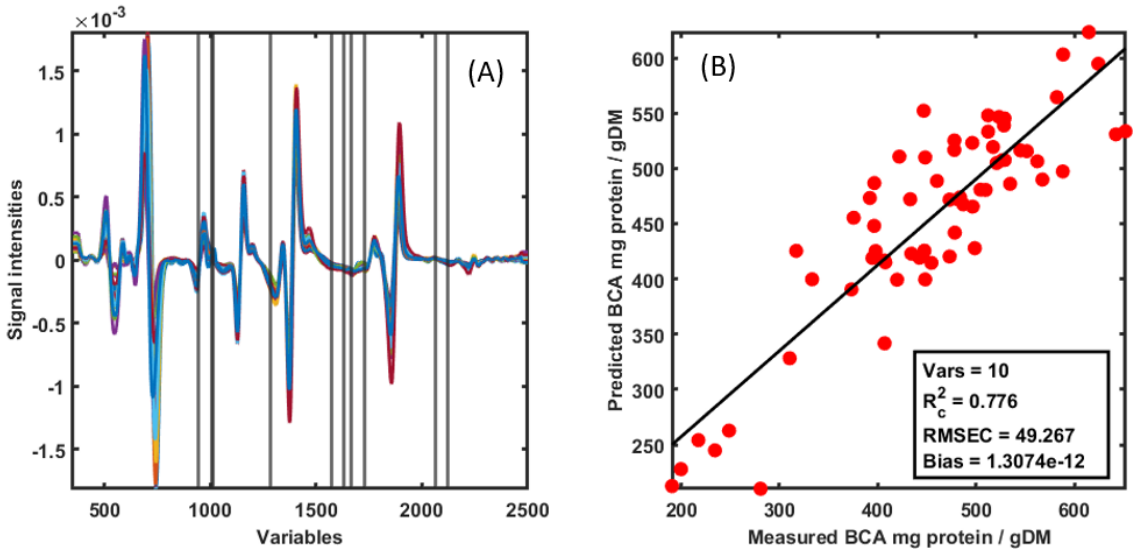


Figure 15 Summary of near-infrared prediction models for protein content. (A) Key wavelengths of interest, and (B) measured vs predicted protein.

Table 2 Summary of key wavelengths selected by the model.

Properties	Dry matter	Protein
Wavelengths	394, 395, 422, 616, 709, 712, 876, 1087, 1221, 1727, 1802, 2264, 2448	944, 1007, 1010, 1285, 1571, 1628, 1666, 1729, 2061, 2122

3.2 Storage experiment effect high volume storage

3.2.1 Storage conditions

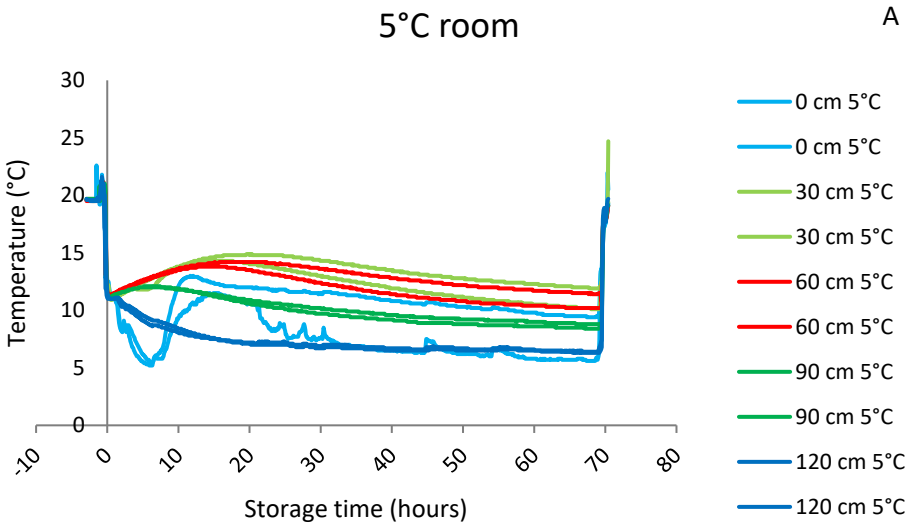
To investigate the effect of storage in higher volumes, sugar beet leaves were stored in large boxes of 1.2 m^3. Sugar beet leaves were harvested manually to limit the wounding areas and the mechanical damages that may occur during harvest and transport. In the field, the leaves were transferred directly into the box. Leaves were allowed to settle based on their own weight without applying additional pressure.

After filling the boxes directly on the field, they were transported to the cooling facility and either covered with a plastic tray when stored at 5 °C (Figure 3-B) or with a plastic film (with some macro-perforations) on the top when stored outside. The temperature and gas contents at the pre-defined layers were monitored at regular intervals (every 5 minutes for temperature and twice a day for gas contents).

The temperature recordings are plotted in Figure 16. For the box stored at 5 °C (Figure 16-A), the temperature of the top layers (0, 30 and 60 cm) increased to 15 °C during the first 24 h; while the bottom layer cooled down immediately. After 50 h of storage, temperature in each layer reached a steady-state condition. The warmest layers were located between 30 and 60 cm from the top of the box, the coldest at the bottom layer, followed by the top layer.

For the box stored for 24 h outside and 48 h at 20 °C, the temperature through the layers was affected by the location of the box. During the first 24 h, a minor rise (7 °C) in temperature of the layers at 30 and 60 cm was observed. For the other layers, temperature followed the outside temperature fluctuation. Once the box was relocated into a climate room at 20 °C, temperature increased in all the five layers. The biggest increase was located in the layer at 60 cm deep, followed by the layer at 90 cm. After 68 h of storage (44 h at 20 °C), the temperature recordings showed that the warmest spot was located at 60 cm depth (centre of the pile) and reached a maximum temperature of 31.9 °C. The temperature exceeded 30 °C for a period of 5.5 h.

More pronounced and faster temperature raising through the storage boxes were expected. The mild packing density applied in the boxes may have allowed some air movement though the pile and/or limited the heat accumulation between the sugar beet leaves.



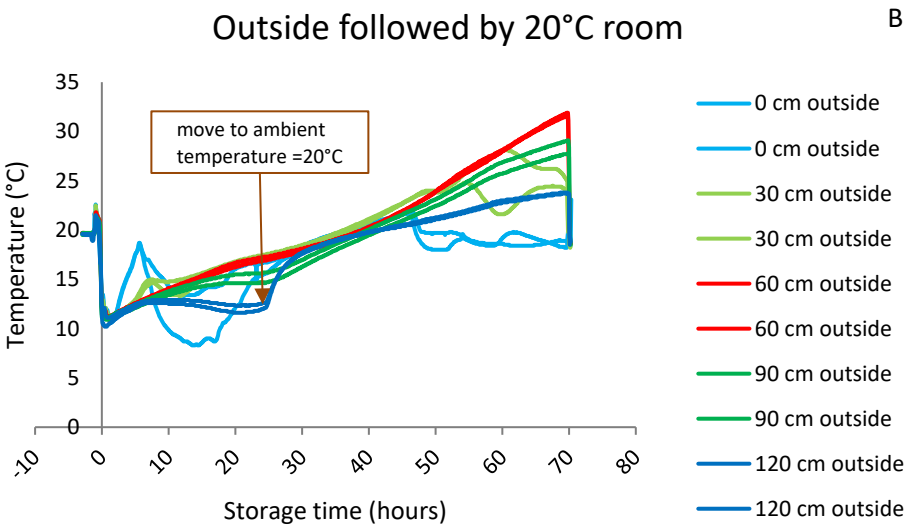


Figure 16 Temperatures recorded through the box of sugar beet leaves stored continuously at 5 °C (A) or stored first 24 hour outside followed by 2 days at 20 °C (B). Layers are identified by the distance from the top of the box. At each layer, two Logtag temperature sensors recorded the temperature.

When looking at the gas contents measured in the different layers of the box, no significant change in oxygen depletion or carbon dioxide accumulation was observed on the box stored at 5 °C (Figure 17-A). This indicates that the respiration rate of the sugar beet leaves was really limited. It seems that gradation of 10 °C between the warmest and coldest layers, combined with the mild packing density, did not allow the O_2 and CO_2 contents to fluctuate sufficiently for the Checkpoint 2 to detect it.
The gas content measurements of the box stored at 20 °C showed clear, but still limited, oxygen depletion and carbon dioxide accumulation in the different layers (Figure 17-B). During the first 24 h of storage, oxygen and carbon dioxide remained constant through the layers; 24 h after moving the box to the 20 °C room, a clear gradation with the oxygen and carbon dioxide was visible. The lower layers (120 and 90 cm) showed reduced O_2 (13 %) and increased CO_2 (8 %) contents. Although the changes in O_2 and CO_2 contents were clearly the results of the respiration rate of the sugar beet leaves, an offset between the layer with the highest temperatures and the ones with the higher CO_2 content can be noted. This may be explained by the mild packing density inside the box. Due to the mid density, gas may have migrated easily from one layer to the other. As the bottom was completely covered with a plastic foil, the increase of CO_2 was more visible at the bottom layers.

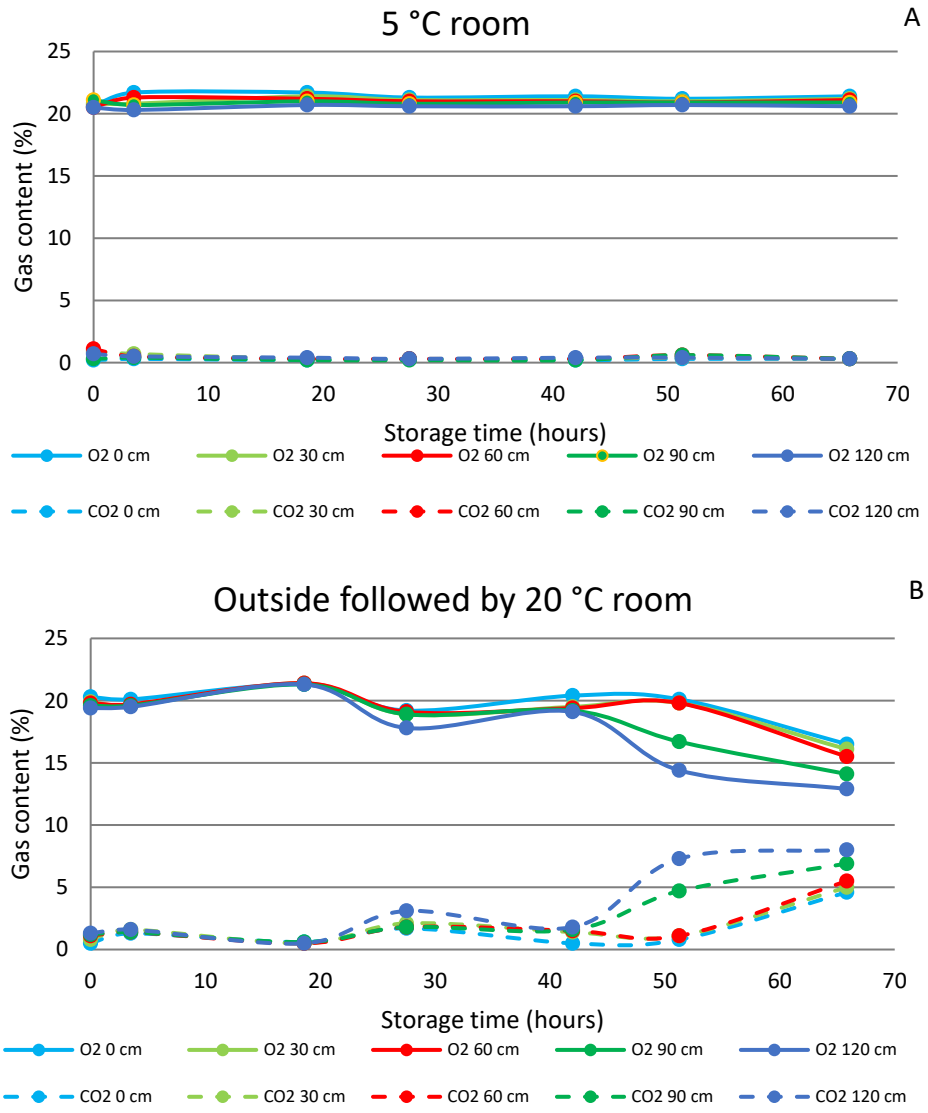


Figure 17 Oxygen (solid line) and carbon dioxide (dash line) contents measured over the time and through the five levels of the boxes stored at 5 °C (A) and outside followed by 20 °C (B).

3.2.2 Dry matter

The dry matter of the sugar beet leave was measured at several stages of the protein extraction procedures. First the dry matter of the leave material was measured following the same procedure that in the previous experiment: half of the leaf, without stem and midribs material, was used for the dry matter measurement. No significant difference between the temperature treatments or between the box layers was observed (Table 3).

After pressing the leaves, the dry matter contents of the juice and the pulp were measured. A significant effect of storage temperature and box layers was observed on the dry matter content of the juice. The juice of fresh sugar beet leaves showed a higher dry matter content when leaves were stored at 20 °C at the top of the box. The lowest juice dry matter content was measured from leaves stored at 20 °C and 90 cm depth.

Concerning the amount of juice extracted per kilogram leaves, leaves stored at 20 °C at the bottom and on the top of the box showed the lowest juice extraction percentage. Although the moisture lost was not measured, leaves collected at these two locations showed dehydration symptoms.

Table 3 Dry matter content of whole leave, juice and pulp extracted of the sugar beet leaves stored at 5 °C and 20 °C in big boxes. The sampling layer in the box is indicated by the distance from the top of the box. The percentage of juice collected from 1 kg of sugar beet leave is given in the last column.

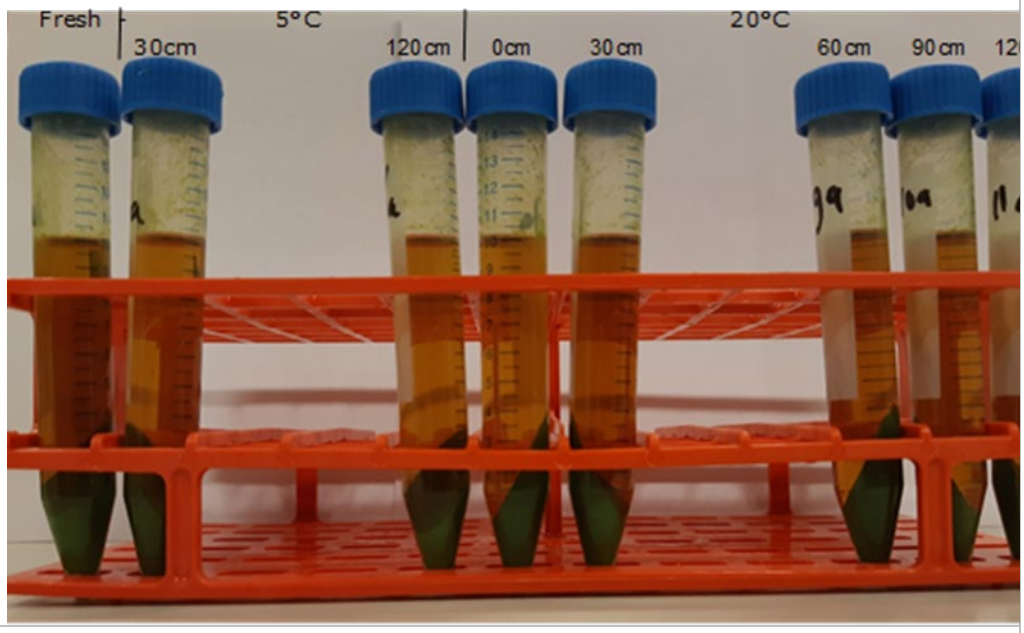
Temperature	Distance	DM (%)			% of juice
		Whole leave	Juice	Pulp	
Start		13.87	9.87 f	27.18	72.5
5°C	30cm	13.87	9.51 c	28.38	73.7
	120cm	13.45	9.58 c	26.00	72.9
20°C	0cm	12.76	9.03 a	24.84	70.8
	30cm	13.46	9.70 e	27.78	71.7
	60cm	14.26	9.39 b	28.50	73.3
	90cm	11.17	9.06 a	28.01	74.8
	120cm	13.31	9.41 b	24.97	70.2

3.2.3 Coagulation

To separate the RuBisCo protein from all other plant cell tissues such as cell membranes, a coagulation step is required in the extraction protocol. The coagulation with the help of addition of $CaCl_2$, heat and centrifugation separate the RuBisCo protein (remaining in the supernatant) from the cell tissue (precipitate). During the pressing process, metabisulfite was also added to the juice to neutralise any binding between phenol and RuBisCo molecules.

Although all these steps are required to extract the RuBisCo from the sugar leave juice, the intensity of the heat step and of the centrifugation step may have an effect on the RuBisCo extraction yield. For this reason, two coagulation treatments were applied on the juice: a standard coagulation protocol (A) and a shorter milder one (B). Figure 18 shows pictures of the coagulated samples after both coagulation protocols. Samples treated with the short coagulation step showed a turbid supernatant. No conclusion about the colour of the supernatant could be drawn based on these pictures, as the colour of the racks (red and blue) could influence the visual colour perception of the samples.

A



B:

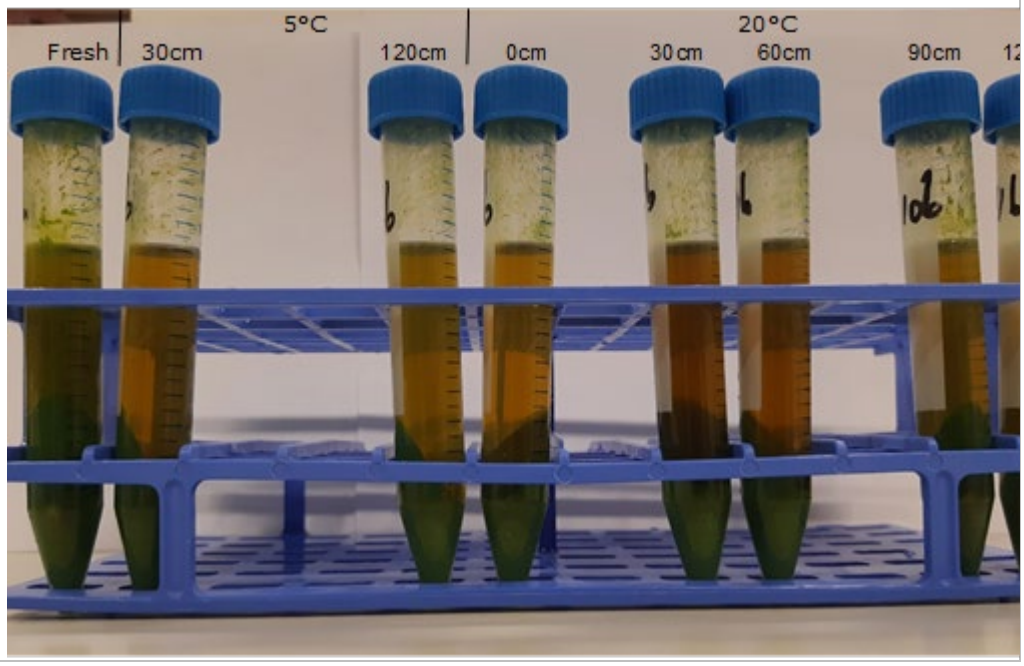


Figure 18 Pictures of the tube after standard (A) and short (B) coagulation treatments of sugar beet leave juice obtained after storage in big boxes at 5 °C and 20 °C and at several layers thorough the box.

3.2.4 Turbidity, chlorophyll and phenol contents

Both the chlorophyll content and the turbidity are indicators of how successful the coagulation was performed. Poor coagulation results in a turbid supernatant with a high thylakoid membrane content, which in turn contains chlorophyll, resulting in a high chlorophyll content.

From the supernatant collected after the two coagulation treatments, the turbidity was analysed. Figure 19 (left) shows the turbidity measured in each sample. Samples treated according to the standard coagulation protocol showed almost no turbidity and no significant differences between the storage treatments were observed. Concerning the samples treated with the short coagulation protocol, Unstored samples (Start) presented the higher turbidity, while samples stored at 30 cm deep in the 5 °C storage room and at 0 cm deep at 20 °C displayed the lower turbidity. No correlation with the dry matter content or the percentage of juice extracted of these samples could be found. The turbidity could, however, be explained by the amount of chlorophyll remaining in the supernatant after

coagulation. The relative chlorophyll content was only measured in the short coagulation samples (Figure 19-right). Similar trends were observed as with the turbidity results: the highest chlorophyll content for the fresh sample followed by samples stored at 20 °C and 120 cm deep.

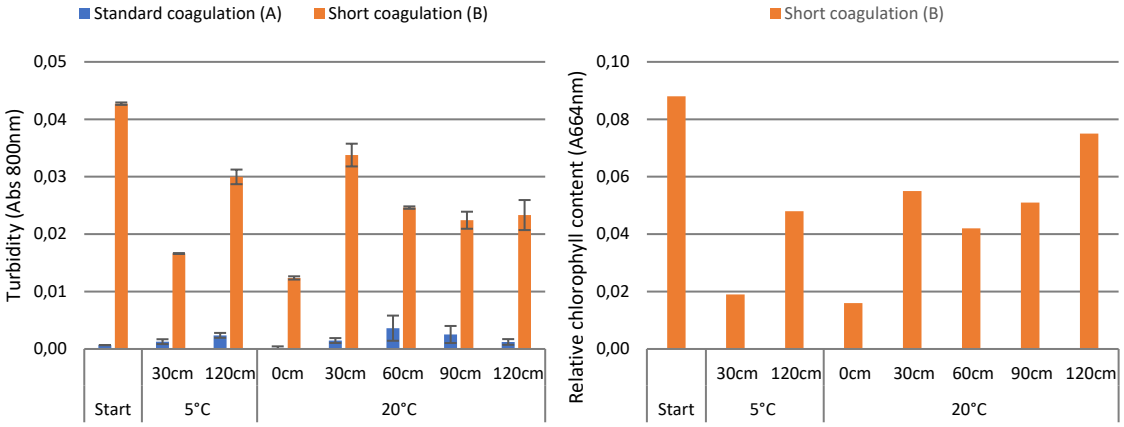


Figure 19 Turbidity of supernatant of sugar beet leave juice after a standard (A) and short (B) coagulation treatment (left figure). Relative chlorophyll content measured in supernatant of sugar beet leave juice after short coagulation treatment (right figure). Error bar represents the standard deviation (n = 2).

The phenol content of these samples was also investigated . Since no sigificant differences between the two coagulation treatments had been observed the phenol results of these two batches had been grouped for the statisctical analysis. Variations in the phenol contents were observed for the samples collected through the several layers of the boxes (Table 4) However, it was not clear if these variations were induced by the different temperatures in these layers. The limited temperature differences, as well as the limited amount of samples analysed (for logistic reason), did not allow to statistically identify the reason of these differences between in the phenol contents.

Table 4 Average absorbance of phenol measured in supernatant of sugar beet leave juice after both coagulation treatments. Different letters in the last column indicate least significant difference between the treatments (n=4; Fischer test; P<0.05)

Temperature	Position in box	Absorbance phenol	L.s.d.
Start		1.162	c
5 ºC	30 cm	0.983	a
	120 cm	1.082	abc
20 ºC	0 cm	1.031	ab
	30 cm	1.179	c
	60 cm	1.086	abc
	90 cm	1.112	bc
	120 cm	1.067	abc

3.2.5 BCA and Bradford analyses

The total protein content was analysed according to either the BCA or Bradford method, depending to the processing method applied to the samples. Samples taken from frozen sugar beet leaves were analysed with the BCA method to compare the total protein content of samples stored in the boxes to the protein contents of the previous experiment. The total protein content of the juice samples was analysed according to the Bradford method, as addition of metabisulfite during the juicing process interferes with the BCA method.

The total protein contents measured on the frozen plant material is summarized in Figure 20. As no information about any weight losses that may have occurred during the storage in the box were available, it was not possible to show the results in mg protein per gram initial fresh weight. No significant differences between the storage temperatures and the position in the storage box was observed for the total protein content.

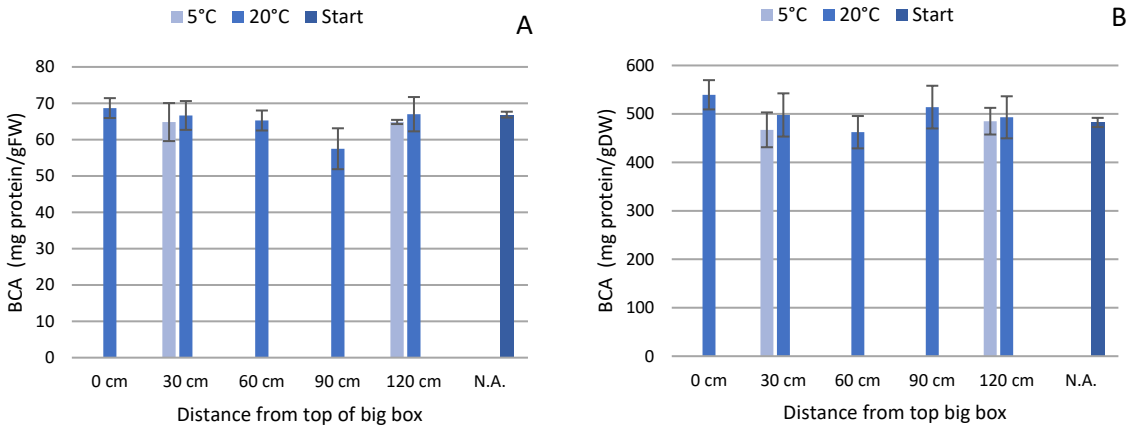


Figure 20 Protein content measured according to BCA method in sugar beet leaves stored at 5 °C and 20 °C in big boxes and sampled at several layers through the box height (distance in cm from the top of the box; N.A.: not applicable for start samples). Protein content is expressed in mg BSA protein per gram initial fresh weight sugar beet leave (A) and in mg BSA protein per gram dry weight (B). Bars indicates the standard error. (n=3).

The total soluble protein content of the juiced samples is expressed in g soluble protein per kg fresh sugar beet leaves (Figure 21-left). For all treatments, samples treated with the short coagulation treatment showed higher soluble protein contents compared to the standard coagulation treatment.

The higher protein content measured by the Bradford assay may result from an artefact effect; the short coagulation treatment was less effective (higher turbidity and chlorophyll content) and by consequence less soluble protein such as very small membrane protein may have remained into the supernatant. These very small membrane proteins were measured by the Bradford assay and accumulated to the soluble proteins. The standard coagulation protocol allowed correct coagulation and better separation of the soluble protein from the rest of the cell tissues. This results in lower soluble protein contents. The highest soluble protein content was measured on the start sugar beet leaves material. This indicates that whatever the storage conditions, the soluble protein content decreases when leaves are not processed directly after harvest. The Figure 21-right shows the soluble protein content yield when comparing to the protein content in the start samples.

Small differences between samples located at different locations through the pile of sugar beet leaves were also observed. However, when regarding only the results obtained after the standard coagulation treatment, no significant difference between the samples was found.

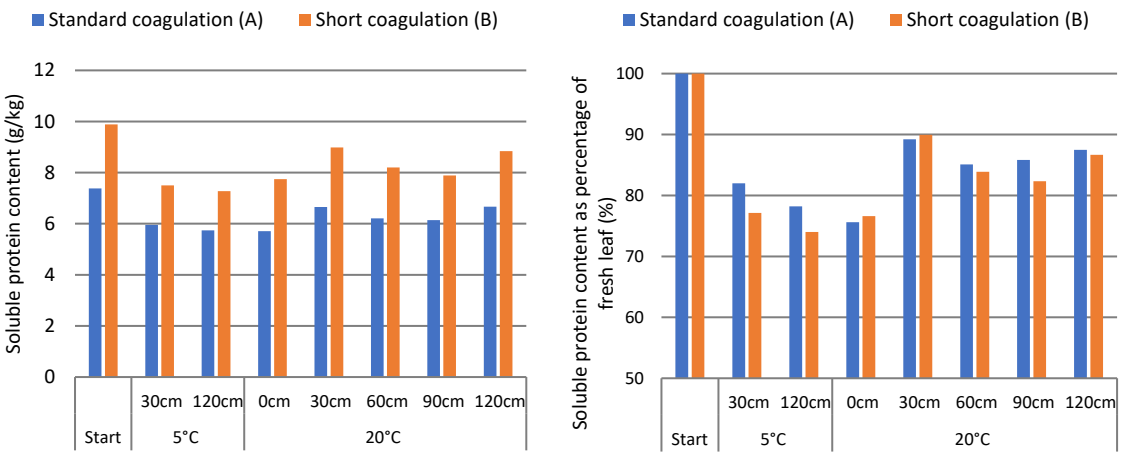


Figure 21 Protein content measured according to Bradford method in sugar beet leaves stored at 5 °C and 20 °C in big boxes and sampled at several layers through the box height (distance in cm from the top of the box). Juice was first extracted from leaves and the standard coagulation treatment was applied prior measuring the soluble protein content. Soluble protein content is expressed in g protein per kilogram initial fresh weight sugar beet leave (A). The soluble protein yield after storage was compared to the fresh material and is expressed in % (B).

3.2.6 RuBisCo content measured with Size Exclusion Chromatography (SEC)

The RuBisCo content was analysed via Size exclusion chromatography (Figure 22). The RuBisCo content showed similar trends as those observed for the total soluble protein contents. The fresh sample extracted from start leave material (no storage) showed a higher RuBisCo content (Figure 23). The short coagulation treatment retained the most RuBisCo compared to the standard coagulation protocol. This may be explained by the shorter heat step applied during the short coagulation treatments: less RuBisCo deteriorated due to the milder heat step. Samples stored at 20 °C deeper in the box (30, 60, 90 and 120 cm) showed slightly higher RuBisCo content than samples stored at 5 °C.

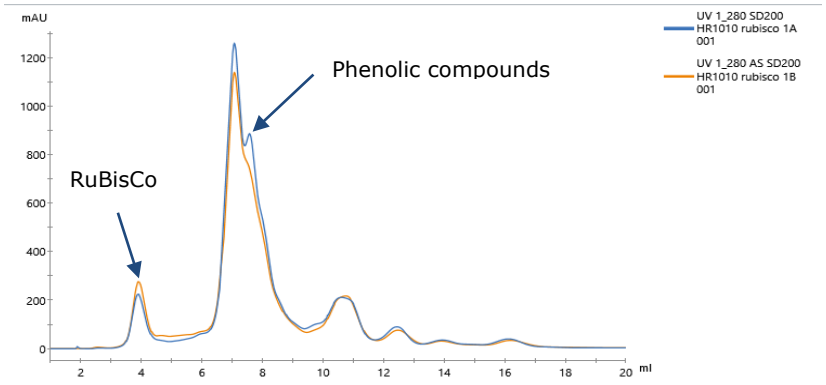


Figure 22 Example of chromatogram obtained after size exclusion chromatography. The peak at 4 ml aligns with the RuBisCo protein. The shoulder peak at 7.5 ml shows the amount of phenol present the sample.

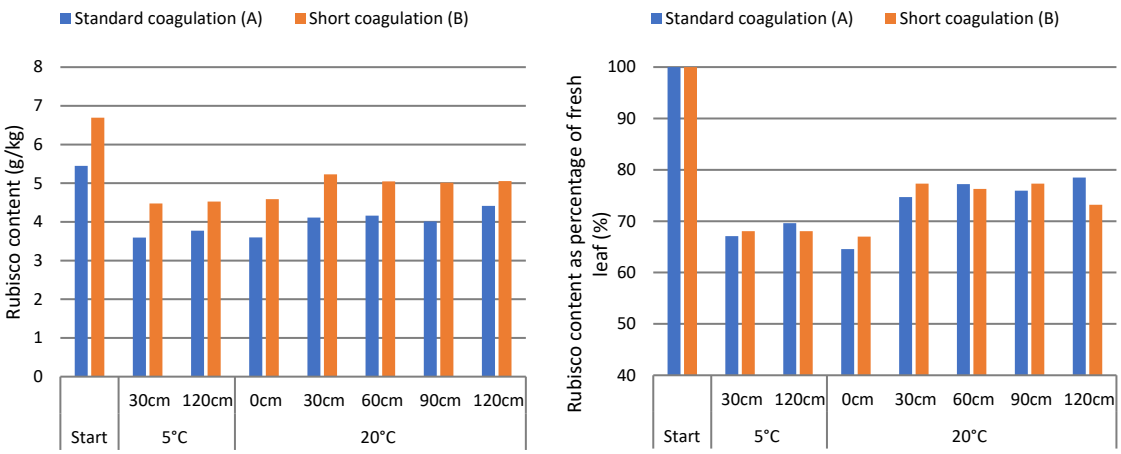


Figure 23 RuBisCo content measured with the size exclusion chromatography in sugar beet leave juice after storage at 5 °C and 20 °C in big boxes. Samples were collected at several layers through the box height (distance in cm from the top of the box). Juice was first extracted from leaves and the standard and short coagulation treatments were applied. RuBisCo content is expressed in g protein per kilogram initial fresh weight sugar beet leave (A). The RuBisCo yield after storage was compared to the fresh material and is expressed in % (B).

3.2.7 Protein gel (SDS-Page)

The integrity of the RuBisCo protein was investigated by SDS-Page gel on samples extracted from the frozen leave material (fresh extraction) and from the sugar beet leave juice (juice extraction). Samples of both extraction methods were loaded on the same gels (Figure 24).

Samples from juice extraction showed two distinctive bands corresponding to the two RuBisCo sub-units: one at 14 kDa and one at 53 kDa. Contrary, samples from fresh extraction showed only one band at 53 kDa. These results for the fresh extraction were consistent with the gel applied on samples collected by previous experiments (paragraph 3.1.5 and Paillart et al. (2020)). The occurrence of the 14 kDa band may result from the different extraction method applied on these samples: protein extraction buffer, addition of metabisulfite added during the juicing process and heat step applied during the coagulation may have played a role to isolate the smaller RuBisCo sub-units on the SDS-gels.

When comparing the two storage temperature treatments and the location of the sugar beet leaves in the box, no clear differences between the gels can be observed. This can be explained by the mild heat period that was applied during the storage of the box at 20 °C. From the first experiment, the RuBisCo degradation was initiated only after 48 h at 30 °C. As the warmest sport in the centre of the pile did exceed 30 °C for only 5.5 h, the storage conditions were too mild to affect the RuBisCo stability. It is recommended for the coming season to repeat the test with a higher packing density in order to reach temperature above 50 °C for a longer period.

Some slots (20 °C at 30 cm replicate B, for instance) showed lighter bands but were not similar between the three replicates. For this reason, no conclusions about the possible effects of storage on the RuBisCo stability can be drawn.

When comparing the gel-slots from the juice extraction, no differences in bands and intensities can be observed. This supported the observations previously done on the other attributes (total soluble protein content, RuBisCo content, …) where no clear effect of the storage could be drawn.

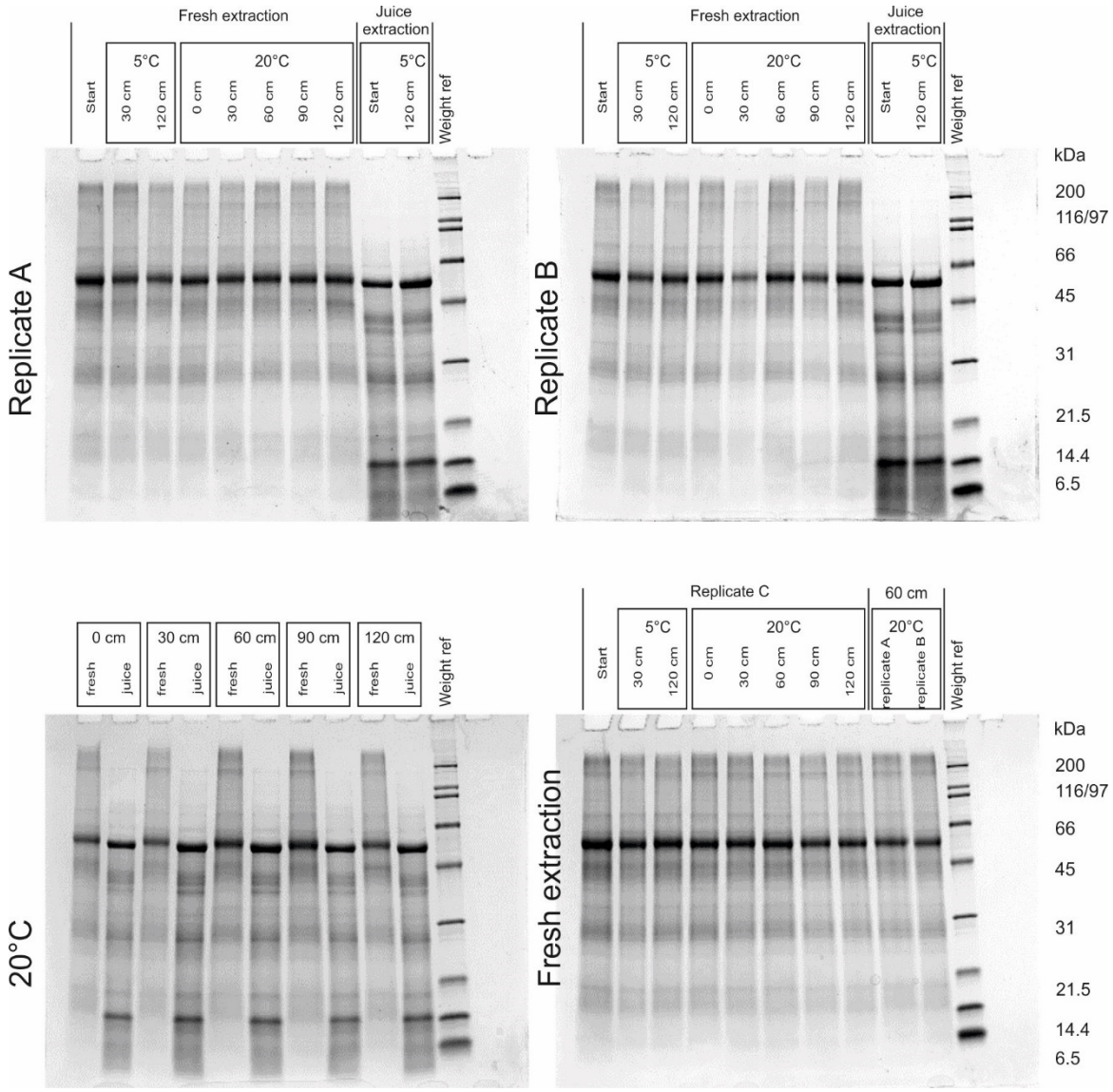


Figure 24 SDS-page gels of proteins extracted from fresh leaves of sugar beet leaves stored at 5 and 20 °C in big boxes for 68 hours. The upper two figures and the lower left show the protein content for each replicates obtained from the protein extraxtion apploied on whole fresh leave. The left lower figure compares the protein extraction of the sugar beet leave stored through the box at 20 °C. For each layer both extractions (fresh and juice) are placed next to each other. Slot on the right side of each gel shows the Low Molecular Weight (LMW) standard going from 6.5 to 200 kDa.

4 Conclusions

During the previous research on storage of sugar beet leaves (2019) reported by Paillart et al. (2020), several conclusions and recommendations were established. The main goals of the tests reported in the present document were to confirm the effect of abuse storage temperatures and to study the effect of storing with higher volume on the RuBisCo stability of sugar beet leaves.

Based on the first experiment, it was concluded that the sugar beet leave can be stored up to 168 h at 1 °C without any major effect in RuBisCo degradation. When sugar beet leaves are stored at 20 °C, loss of RuBisCo is initiated after 72 h of storage, 48 h at 30 °C and between 24 and 30 h when leaves are subjected to a temperature of 45 °C.

The second part of the research was based on testimony of other researchers that had assumed a correlation between the fast RuBisCo degradation and extreme temperature rising inside pile of sugar beet leaves after harvest. The storage conditions applied during the experiment did not manage to reach these extreme temperature raisings. By consequence, conclusions about the stability of total soluble protein, and RuBisCo in particular, can only be made regarding a mild storage condition, where storage temperature reached 31.9 °C for a really short period of time.

The total protein content and RuBisCo stability analysis on samples extracted on the sugar beet leaves materials showed no significant difference between the two storage temperatures and the different layers through the piles. When regarding to the protein results obtained on the sugar beet leaf juice, no effect on the mass balance and dry matter contents were observed. For all the storage conditions, a decrease between 10% and 25% of total soluble protein content was measured.

When storing the plant material in similar packing density in pile, sugar beet leaves can be stored for a period of 68 hours without any major effect on the RuBisCo stability:

- Storage in pile (big box) in a cool room set up at 5 °C helped to avoid temperature rising through the pile.
- RuBisCo was stable while temperature inside the box remained under 30 °C for the majority of the storage period (62.5 h).

Based on these observations, it is recommended to further investigate the effect of temperature rising through a pile of sugar beet leaves with higher packing density (similar to the ones applied at industry level).

Predictive models based on dry matter and on non-destructive sensing technologies were also investigated: the correlation between the dry matter and the protein content was judged poor and indicated that it is not possible to extrapolate the protein content of the sugar beet leaves on basis of their dry matter content. However, a relatively good performance was achieved for the prediction of the dry matter on basis of 13 wavelength measurement (VISNIR). Unfortunately, the prediction of protein content was less sensitive when using NIR measurement (10 wavelengths). Further measurements are required to optimise this model improve its prediction score (R^2).

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