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

Part II: Yellow pea

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Institute: Wageningen Food & Biobased Research

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Summary

Wageningen University & Research is looking for ways to increase the availability and diversity of proteins. Plant proteins are an attractive alternative to animal proteins. It is important to optimize the recovery methods of proteins from plant based biomass sources. This includes the need to study the effects of post-harvest conditions on the yield and quality of extracted proteins.
There is also an increasing interest in pea as protein source. Therefore, the effects of post-harvest conditions were studied on protein yield and quality in dried yellow peas. The peas were stored under two different temperatures (12 and 40 °C) combined with three relative humidity (RH) levels (range 26 to 86% RH) during four different storage periods (1, 2, 4 and 6 weeks). This led to differences in moisture content after 6 weeks, ranging from 4.8% at high temperature in combination with low RH till 17.6% at low temperature in combination with high RH.

Analyses were done on protein content, protein composition, glycation, hexanal and saponins. Glycation of pea proteins is relevant for the possible improvement of techno-functional properties, such as solubility and interfacial properties. Hexanal and saponins are relevant components in peas which may cause or indicate off-taste in extracted pea protein. The main results of this study are as follows:

- Overall, there was no clear effect of storage condition (temperature, RH) and storage period on protein content, as shown by BCA assays.
- Storage conditions and storage period did not affect protein quality in terms of protein composition, as shown by SDS page.
- We found no evidence of an effect of storage on the off-flavour compound hexanal.
- The relative levels of saponins were measured, but besides potentially small changes at 40°C/85%RH, the storage conditions tested did not seem to have an influence on saponin content and composition.
- We found differences in glycation between samples. However, the results were not consistent. Nevertheless, it is an interesting finding that glycation can be affected in some way.

The described results and conclusions are valid for yellow peas (whole seeds) under the tested circumstances.

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.

For additional information about this report, see the colophon.

1 Introduction

This report is a continuation of the work in the project 'Proteins from plant-based biomass: effects of post-harvest conditions on proteins and quality'. The interest in pea proteins as an alternative to soy proteins has increased due to their amino acid profile, low allergenicity, and high availability (Lam et al., 2016). However, their application in food products still faces challenges in terms of limitations in functionality and flavour/colour issues.

In this report we describe a storage experiment with dried yellow peas. The effects of storage time, temperature and humidity are studied on protein content and aspects of protein quality, including protein functionality. The choices for the storage conditions during the experiment and subsequent analyses were justified as follows.

Storage conditions

In general, peas are considered dry enough for storage in practice when they have less than 16% moisture content (Barker, 2018). The storage length of pea decreases if seed moisture or temperature rises. The following table shows the big influence of temperature and moisture content on storage length for seed peas (Table 1).

Table 1 Number of weeks for safe storage of peas at the specified seed moisture content and storage temperature. Source: Sokansani, 1995 (Barker, 2018).

Temperature (°C)	Moisture Content of Seed (%)				
	12	14	16	18	21
	Maximum Safe Storage (Weeks)				
26	31	16	7	4	2
20	55	28	13	7	4
16	100	50	20	12	6
10	200	95	38	20	21
6	370	175	70	39	20

In our study we focus on finding answer to the question whether there is an effect of storage time, temperature and relative humidity (RH) on protein content and quality. Therefore we included different storage periods (1, 2, 4 and 6 weeks). We have chosen 2 temperatures (12 °C and 40 °C) combined with 3 humidity levels (target 25%, 60% and 80% RH). In this way, we achieved a wide range in conditions, and still practically realistic.

Protein content (protein extractability)

Pulses (including peas) are an interesting source of plant proteins with a high initial protein content that can be more than 24g protein/100g dry matter (Schutyser et al., 2015). Practically relevant is the yield after extraction. A challenge for the industry is to increase this yield of proteins. One possible solution is to breed and grow peas with a higher protein content. Another is to optimize the extraction. Interestingly, however, the effect of post-harvest conditions on protein extractability (and quality) is not known by the industry. The proteins are considered stable after harvest. We have applied different storage conditions to evaluate this.

Protein quality

The protein product derived from yellow peas is of rather high quality, meaning it has most essential amino acids. A great benefit is that it has a "clean label" image, is non-allergenic, and does not contain gluten, making it preferred by many consumers over soy, wheat, lupin, and milk proteins. Another benefit is that it is non-GMO. Also the protein has promising functional qualities: good solubility, viscosity, and emulsifying properties. (Mulder, 2016).

Protein functionality

The interest in pea proteins as an alternative to soy proteins has increased due to their amino acid profile, low allergenicity, and high availability (Lam et al., 2016). However, their application in food products is still limited mainly by their limited techno-functionality. Glycation of (pea) proteins with reducing polysaccharides via the first step of the Maillard reaction is able to alter (improve) their techno-functional properties such as emulsifying ability, solubility, as well as heat and pH stability (Oliver et al., 2006; Rao et al., 2018; Kutzli et al., 2020). Glycation may also reduce food allergenicity (Rao et al., 2018). The conjugation of sugar moiety to protein is a process occurring during storage of powders, but the process should not take place on purpose. Otherwise, this process may be considered as novel foods. Therefore, we find it very relevant to study whether storage conditions affect the glycation in yellow peas.

Side-effects of pea protein extraction process

Two main challenges are mentioned by industry for the extraction of yellow pea proteins. The first one is off-taste, which is sometimes a problem. This is associated with the presence of, amongst others, saponins. Saponins are naturally present in peas and others seeds. Peas contain mainly two types of saponins, namely saponin B and DDMP saponin (Heng et al., 2006), also known as soyasaponin Bb and soyasaponin βg, respectively (depicted in Figure 1). The latter names are more distinct, as there are several different B saponins and DDMP saponins. Soyasaponin βg ("DDMP saponin") is the main saponin in untreated peas and has a slightly higher bitterness than soyasaponin Bb ("saponin B"). Soyasaponin βg is sensitive to heat and processing and during handling it is (partially) degraded into soyasaponin Bb, which is slightly less bitter compared to soyasaponin βg (Heng et al., 2006). Besides bitter, saponins are also said to be astringent (Price et al., 1985; Gläser et al., 2020) and metallic (Price et al., 1985).

The second challenge is undesirable aroma, such as "green notes" caused by volatile products (e.g. aldehydes). Many volatile off-flavour compounds originate from enzymatic fat oxidation (lipoxygenases catalyses oxidation of polyunsaturated fatty acids (PUFAs) when they react with the substrate during milling). Furthermore, autoxidation and photooxidation can happen. Hexanal is a volatile compound that is produced when fatty acids are oxidized. The off-flavour intensity of hexanal itself is limited, but it serves as marker compound for other off-flavours generated by oxidation, mainly undesirable aldehydes. Off-flavour is rather difficult to remove by processing, when it occurs. In our experiment, we tested the effect of storage conditions on both hexanal and saponins, as indicators for undesirable taste and aroma (off-flavour). Hexanal is a volatile organic compound while saponins are non-volatile.

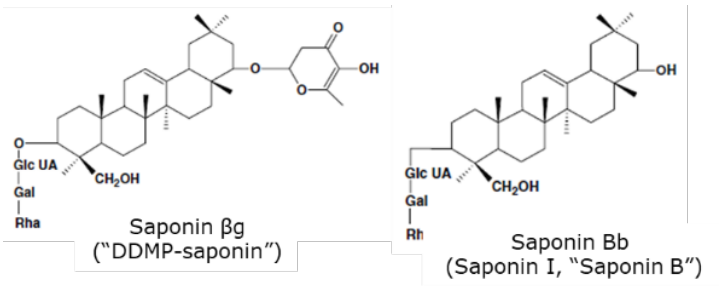


Figure 1 Structures of pea saponins.

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 want 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 Materials and Methods

2.1 General set-up

Dried yellow peas (whole seeds) were stored under different conditions during different periods of time, after which the protein content and quality were analysed. The main research questions and analysis methods are described in Table 2.

Table 2 Main Research questions and analysis methods.

Research question	Method
Do storage conditions/time affect the yield of water extractable protein (% protein) ?	BCA protein assay
Do storage conditions/time affect protein quality, in terms of protein composition ?	Gel electrophoresis (SDS-PAGE in reducing and non-reducing conditions)
Do storage conditions/time affect protein glycation (which is able to improve techno-functional properties such as emulsifying ability) ?	The gel obtained above will be stained with PAS staining
Do storage conditions/time affect protein quality, in terms of off-flavour	1. Hexanal (volatile compound) by GC-MS 2. Saponins (non-volatile compounds) by LC-MS

The storage took place in the period 31 January 2020 – 13 March 2020. The peas had been stored already for several months before the experiment started. This set-up complies well with the practice where companies can also start processing later in the season (first finishing processing of other products like potato).

The peas were stored under two different temperatures combined with three relative humidity (RH) levels during four different storage periods, resulting in 24 treatments. Each treatment was carried out in 4 replicates. The general experimental set-up was therefore as follows: Temperature (2) x RH (3) x period (4) x replicates (4) = 96 objects.

After the different storage periods, peas were removed from storage and several analyses were done:
- Pea weight change (also referred to in this report as 'fresh weight change')
- Dry Matter
- Colour
- Proteins yield
- Protein composition
- Glycation (only for selected conditions)
- Hexanal (only for selected conditions)
- Saponins (only for selected conditions)

Further details are described in the next paragraphs.

2.2 Plant material

Dried yellow peas, cultivar Angelus (spring peas) were kindly provided by APPO in Gembloux, Belgium. They were harvested at the Experimental Farm of the University - Gembloux Agro-Bio Tech and stored in an agricultural hangar under regular storage conditions. The material arrived at WFBR in a closed plastic bag on 29 January 2020 and was stored at 20 °C until the start of the experiment on 31 January.

Damaged peas were excluded from the experiment. For each object, a plastic cup was filled with 35 gram peas. The exact weight was measured (Figure 2).

Figure 2 ***Weighing of the peas into plastic cups at the start of the experiment.***

2.3 Storage conditions and storage period

Peas were stored in plastic cups in two temperature controlled rooms (12 °C and 40 °C) inside stainless steel containers (70 L). These containers were connected to a flow-through system (Figure 3). In this flow-through system, pure N_2 and O_2 from gas cylinders (dry air) were mixed using mass flow controllers to reach 21% O_2, 0% CO_2, and N_2 as balance gas.

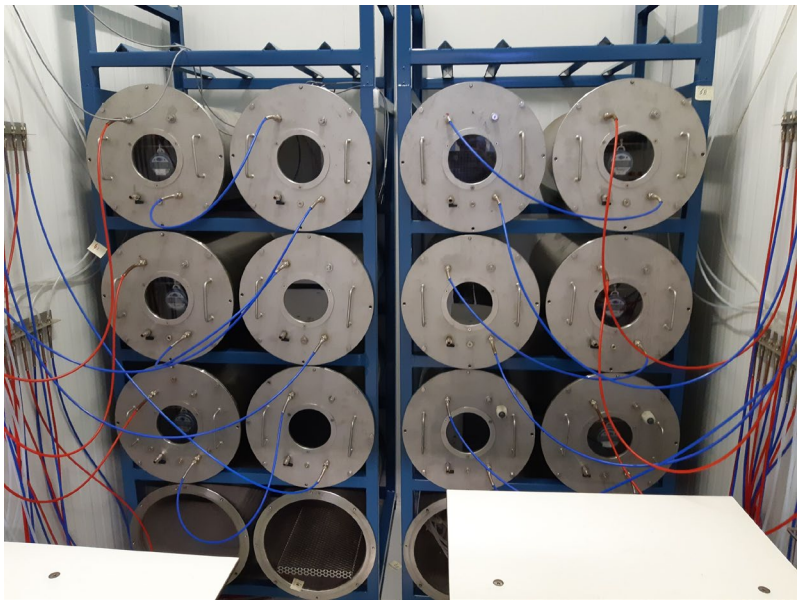


Figure 3 ***Flow-through system with containers which held the cups with peas inside. Air flows of different humidity were led via the tubing through the containers.***

For each temperature, three different RHs were applied. In this report these are indicated as low (target 25% RH), middle (target 60% RH) and high (target 85% RH). These three RH conditions were each applied to 4 containers (indicated as 4 replicates), by which 2 parallel gas flows were led through 2 containers in series. A temperature/RH logger was placed inside the first container of each series. Because of the small amount (120 g) of dried peas per container, combined with high air flow (1000 $ml.min^{-1}$), it can be assumed that conditions of the two containers within a series were equal.

Each container contained 4 plastic cups with peas for either 1, 2, 4 or 6 weeks storage. At these four times the storage containers were briefly opened to remove 1 cup per container.

The various RHs were achieved by combining a dry and a humidified gas flow in different ratios before entering the container (Table 3). Hereby, humidified gas was created by directing the original dry gas through a water flask, resulting in a relative humidity close to saturation. Total flow rate of the combined flows entering the containers was always close to 1000 ml.min^{-1}. The realized RHs and other air moisture conditions were different between the two temperatures (Table 3).

Table 3 Experimental set-up and the realized storage conditions.

Temp. (°C)	Flow of humidi-fied air (ml.min^{-1})	Flow of dry air (ml.min^{-1})	RH (%) Target	RH (%) rea-lized	Stora-ge in weeks	Calcu-lated vapor-pressure deficit (Pa)	Calculated absolute air humidity ((gwater)/(kg dry air))	Repli-cates
12	250	750	Low: 25	**31**	1,2,4,6	968	2.7	4
12	600	400	Mid: 60	**64**	1,2,4,6	505	5.5	4
12	850	150	High: 85	**86**	1,2,4,6	196	7.5	4
40	250	750	Low: 25	**26**	1,2,4,6	5461	11.8	4
40	600	400	Mid: 60	**59**	1,2,4,6	3026	26.9	4
40	850	150	High: 85	**76**	1,2,4,6	1771	34.6	4

2.4 Analysis

2.4.1 Initial measurements starting material

At the start of the experiment, 4 random samples of approximately 10 grams were taken from the initial batch. Fresh weight and dry matter of these samples were determined by the same methods as described in the following paragraphs for stored peas.

2.4.2 Pea weight (‘fresh weight’)

The pea weight per cup was determined at the start of the experiment (±35 grams), and again directly after removing the cups from the storage containers after 1, 2, 4 or 6 weeks (Mettler Toledo type MS403TS/00 scale). Pea weight loss (%) over the storage period was calculated from these data. The pea weight is also referred to in this report as ‘fresh weight’.

2.4.3 Dry matter

After the measurement of fresh weight, approximately 10 grams per cup was taken to determine dry matter content. The samples were weighed and transferred to (pre-weighed) pergamon paper bags. The bags with material were subsequently placed in a drying oven at 80°C and weighed again after 4 days. Dry matter content was calculated from the weighing data.

2.4.4 Colour

Colour images were taken after transferring part of the sample into alumina cups (1 layer of peas). The images were taken 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 using standardized settings.

2.4.5 Proteins

2.4.5.1 Sample preparation for protein extraction

From each fresh pea sample, a duplicate of approximately 10 gram was frozen in liquid nitrogen and subsequently stored in 50 ml costar tubes at – 80 °C until protein analyses started.
Of the selected treatments, mostly two out of the 4 stored replicates were used for protein extraction experiments. The required amount of pea samples was ground using an IKA analytical mill (IKA A11, IKA-Werke GmbH & Co., Staufen, Germany) under liquid nitrogen. The flour was stored at -20°C before analysis.

2.4.5.2 Protein extractions

For protein extractions, 20 mg of frozen ground material was weighed into a 2 mL Eppendorf tube. Two extraction methods were used:

- For most analyses an extraction method was used for disruption and solubilized total protein in plant tissues (Algrisera, AB, Vännäs, Sw).
  The stock (4x) protein extraction buffer PEB, contains 40% glycerol, Tris-HCL pH 8.5, LDS and EDTA. For extraction a freshly ready-to-used PEB 1x was prepared with pH between 8.25 -8.7. A protease inhibitor (Complete®; Roche, Basel, CH) was added to a final concentration of 0.1 mg/ml extraction liquid.
  Extraction of the protein was performed under liquid nitrogen with a MM301 Vibration Mill (Retsch GmbH, Haan,D.). A 3 mm tungsten carbide beat was added to the extraction tube to assure complete cell tissue disruption during shaking. The samples were shaking in stop steps of 2 min at 30 Hz) and centrifuged (Eppendorf 5420, 10.000 x g for 10 minutes) to remove insoluble material. Supernatants were carefully transferred to new tubes and centrifuged one more time 5 min. 10.000 x g. The clear supernatants were aliquoted in three 0.5 µl tubes and directly frozen and stored at -20°C until analysis.

- For the 3rd glycation experiment, for comparison to the standard extraction method, an extraction liquid was used for total proteins under reduced conditions.
  20 mg ground pea flour was directly extracted in 650 µl SDS-PAGE sample buffer with reduction (62.5 mM Tris-HCL pH6.8; 2% w/v SDS; 5% w/v/ 2-mercaptoethanol; 10% glycerol; 0.02% bromophenol blue). The extraction liquid was added while stirring the tube and heated for 30 minutes at 99°C in a thermomixer (Eppendorf). The samples were cooled down to room temperature and centrifuged (5 min, 20.000x g). The clear supernatant was used for analysis.

2.4.5.3 Protein content (BCA assay)

Soluble protein content of recovered supernatant was measured using the modified Biuret method (Pierce™ BCA protein assay kit). This method is compatible with detergents e.g. LDS.
One aliquot of the frozen supernatant (2.4.5.2.) was thawed, mixed and diluted with an 0.9% sodium chloride solution to fit the calibration curve (0.1 – 1,2 mg protein/ml). The protein bovine serum albumin (BSA) was used as reference. The assay was performed in a 96-well microplate and the absorbance at 562 nm was measured in triplicate. The average was taken to compare the protein content between the different storage treatments.

2.4.5.4 Protein composition (SDS-PAGE)

Characterization and changes in protein composition of the supernatants were examined with reducing and non-reducing SDS polyacrylamide gel electrophoresis (SDS-Page) using a Bio Rad Mini-Protean cell (Bio-Rad Laboratories). For reducing SDS-PAGE samples were diluted with sample buffer 2x (125 mM Tris-HCL pH6.8; 4% w/v SDS; 10% w/v/ 2-mercaptoethanol; 20% glycerol; 0.04% bromophenol blue) to equal protein concentration. The protein content measured by BCA method was used to dilute the protein concentration of all samples to 2 mg/ml. For non-reducing condition sample buffer without 2-mercaptoethanol was used. After dilution the samples were heated for 5 minutes at 99°C (thermomixer) and centrifuged 5 minutes at 12.000x g. An amount of 5 µl of each sample (1 µg protein) was applied on an AnyKDa Tris-HCL SDS-ready gel and electrophoresis was carried out at 180V for about 50 minutes. 6 µL of molecular protein standard (LMW-B) from Bio-Rad (Broad range 0f 6.5 to 200 kDa) was applied as reference for protein characterization.

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

Coomassie brilliant blue staining

After electrophoresis the proteins were stained with Coomassie Brilliant Blue R250 stain solution (0.2% in 40% methanol, 12% acetic acid) for 30 minutes at 40 °C. The gels were de-stained with a solution of 10% methanol, 10% acetic acid at room temperature

Glycoprotein staining

To detect the presence of the sugar moieties in the protein bands, the periodic acid-Schiff's staining (PAS) method was used (Glycopro -Sigma). After electrophoresis the gels were soaked in a fixing solution followed by washing, oxidation, staining and reduction steps according the standard protocol. Horse radish peroxidase (HPR) was used as a positive reference.

2.4.5.5 Glycation

1st experiment : influence of storage condition

A subset of samples was analysed to get a first impression of the relevance of storage conditions for glycation. One of the 4 replicates of storage containers was used for this exploratory experiment. Samples used were:

- Starting material (week 0) (1 sample)
- 12 °C, low RH : 1 week, 2 weeks, 4 weeks, 6 weeks storage (4 samples)
- 12 °C, high RH: 1 week, 2 weeks, 4 weeks, 6 weeks storage (4 samples)
- 40 °C, low RH : 1 week, 2 weeks, 4 weeks, 6 weeks storage (4 samples)
- 40 °C, high RH: 1 week, 2 weeks, 4 weeks, 6 weeks storage (4 samples)

Protein extraction was done according to the standard protocol (2.4.5.1) and total protein in the supernatant was measured using the BCA protocol (2.4.5.3).
Changes in protein composition were examined with reduced Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the anyKDa gel system tris/glycine/SDS.
Gels were stained for protein (Coomassie Brilliant Blue method 2.4.5.4.1) and glycation (2.4.5.4.2).

2nd experiment: pea samples with/without hull

A small set of samples was analysed to investigate whether the observed glycation in the 1st experiment would still be found only for the cotyledons, after dehulling. Therefore, part of the peas were also dehulled before grinding. The following 6 samples were analysed:

- Starting material (week 0) (1 sample with hull, 1 sample without hull)
- 12 °C, low RH : 1 week storage (1 sample with hull, 1 sample without hull)
- 12 °C, high RH: 1 week storage (1 sample with hull, 1 sample without hull)

The samples of 12 °C were from the same replicate as in the 1st experiment.
Protein extraction was done according the standard protocol (2.4.5.1) and total protein in the supernatant was measured using the BCA protocol (2.4.5.3.) Protein concentration applied on to gel was 1 µg protein.

Changes in protein composition were examined with reduced Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the anyKDa gel system tris/glycine/SDS.
Gels were stained for protein (Coomassie Brilliant Blue method 2.4.5.4.1) and glycation (2.4.5.4.2).

3rd experiment: 2 different protein extraction liquids
Based on the results of the previous experiments, it was also decided to study the extraction for the glycoproteins under reduced condition compared to the extraction of glycoprotein in the presence of a protease inhibitor. The following 6 samples were analysed:

- Starting material (week 0) (1 sample with hull, 1 sample without hull)
- 12 °C, low RH : 1 week storage (1 sample with hull, 1 sample without hull)
- 12 °C, high RH: 1 week storage (1 sample with hull, 1 sample without hull)

These samples all came from a replicate other than used in the 1st and 2nd experiment.

4th experiment: Differential Scanning Calorimetry (DSC)
Glycation of the proteins (glycoproteins) may affect the physical property of the proteins. Thermal stability, with Td as an indicator, was evaluated by Differential Scanning Calorimetry (DSC).
Thermal properties of globular proteins are important, since they can be related to their heat-induced aggregation and gelation behaviour. A higher denaturation temperature (Td) is usually associated with higher thermal stability for a globular protein. The Td can also reflect the disruption of hydrogen bonds that maintain tertiary and quaternary structures of proteins, particularly tertiary ones. Thus a higher Td for the proteins would suggest that the polypeptides have a more compact tertiary structure.
For this purpose 6 samples were analysed, the same as used for the 3rd experiment.

Preparation of pea protein solution:
Because pea flour consists of approximately 20% proteins and 45% starch, we first remove the starch. Therefore, the flour was suspended in demi water, buffered with Tris/HCl pH 8 with 0.2 M NaCl (0.25 gr flour/ml). The proteins were extracted at room temperature for 1 hour on a rolling bank. Suspensions were centrifuged 10 min, 5000x g at 10°C to remove the starch and the supernatant was used for DSC analysis.

DSC:
Thermal transition of the six pea protein samples was examined by DSC using a Q200 (TA Instruments, USA). Of each protein extract, approximately 30 mg of the liquid sample was weighed into an aluminium liquid pan. Pans were hermetically sealed and heated from 10 to 160 °C at a rate of 5 °C/min. A sealed empty pan was used as a reference. The denaturation parameters Onset temperature (To), peak transition or denaturation temperature (Td) and transition enthalpy (delta H) were calculated from the thermograms by Universal Analysis 2000, Version 4.1D (TA Instruments Waters LLC).

2.4.6 Hexanal (marker for volatile off-flavour)

Hexanal was measured to get an idea about the development of off flavours caused by oxidation during storage. Hexanal measurements on GC-MS were done from the headspace above the samples, both on milled dry samples (1 g; dry method) and on samples in a watery solution (1 g/2ml water; wet method). The analyses of the dry samples show the "oxidation" pea flavour perceived above the dry samples. The analyses of the wet samples represent the flavour perceived when the material is in moist conditions.
These analyses were done on a part of the samples. Besides starting material (week 0), samples from 12 and 40 °C after 6 weeks storage at 25 and 85% RH (target RH) were analysed. For this exploratory experiment, two out of four storage container replicates were used.

GC-MS conditions
A Thermo GC-MS system, consisting of a GC1300 and a ISQ7000 single quadrupole MS was used. The cold trap was set to -130°C, holding time 2min, and contained a 0.32mm pre-column. The GC-column used was a Rxi@-5Sil MS, 30m x 0.25mmID, 1µm df (Restek). The volatile extraction was performed with HS-SPME-ARROW 1.2 µm DVB/CarbonWR/PDMS. Analyses was done with GC-MS. The incubation

time of the GC vial was 2 minutes, the extraction time was 20 minutes, and the desorption time was 2 minutes. The relative area of hexanal was determined and reported.

Dry method
1 gram of milled peas (milled in liquid nitrogen in order to prevent heat development) was put in a 20 ml vial. The compounds in the vial were incubated 40°C for 2 min, extracted at 40°C for 20 min with HS-SPME-ARROW and the SPME-Arrow was desorbed for 2 min prior to GC-MS analysis. The relative area of hexanal was determined and reported.

Wet method
1 gram of milled peas (milled in liquid nitrogen in order to prevent heat development) was put in a 20 ml vial and 2 ml water was added. The vial was closed and shaken for 5 min with a vortex mixer. The rest of the procedure was identical to the dry method.

2.4.7 Saponins

Saponins were analysed with a semi-quantitative method (comparison between samples).
In analogy to the GC-MS measurements, the HPLC-MS measurements were done on a part of the samples. Besides starting material (week 0), samples from 12 and 40 °C after 6 weeks storage at 25 and 85% RH (target RH) were analysed. For this exploratory experiment, two out of 4 storage container replicates were used.

Material
As analytical standard, soyasaponin Bb was used (Phytolab 86545, purity 98%). Saponin Bb was dissolved in methanol / water 50/50 (v/v%) at a concentration of 10.72 mg/L. It was measured at 1.072 mg/L, 0.1072 mg/L, and 0.01072 mg/L. At 0.01072 mg/L, saponin Bb was not detectable anymore. All HPLC analyses were performed in duplicate.

Methods
1) Preparation of HPLC samples
Peas were ground under liquid nitrogen. Grinding of the peas was done freshly for samples 44:40-85-C and 48:40-85-D just before extraction and analysis, due to lack of already ground material. For all other samples, peas already ground some months earlier were taken from the freezer and defrosted before extraction. 200 mg of the ground peas were extracted with 10 mL of methanol / water 50/50 (v/v%) by stirring for 1 hour. The solids were removed by filtration. The pea extract was used for HPLC analysis. After pea area integration, the area was corrected for the exact weight of the sample used for extraction.

2) HPLC-MS analysis
An Agilent HPLC-MS system, consisting of an autosampler, column oven, and gradient pump (all 1200 series) and a mass spectrometer (6410 Triple quad LC/MS), was used.

a) HPLC

Sample separation was carried out with a XBridge Shield RP18 (150 mm x 4.6 mm, 3.5 µm) HPLC column (Waters art. no: 186003045) and a XBridge BEH Shield RP18 VanGuard Cartridge (5 mm X 3.9 mm, 3.5 µm, 130Å) pre-column (Waters art. no.:186007804) (both Waters, Ireland). The solvent system consisted of mobile phase A: water-acetonitrile-formic acid (99-1-0.1% (v/v)) and mobile phase B: acetonitrile-formic acid (99.9-0.1% (v/v)). Each injection had a volume of 15 µL. The flowrate was 0.4 ml/min, the temperature of the column oven was 30 ˚C, the temperature of the autosampler tray was 10 ˚C in the dark. The elution program was as follows: 0→60 min, 10→100% B (linear gradient); 60→61 min, 100→10% B (linear gradient); 61→66 min, 10% B (isocratic).

b) MS

The triplequadrupole MS was used in positive ion mode with a capillary voltage of 4000V, gas temperature of 350°C, gas flow of 10 L/min, and nebulizer of 55 psi.

Full Scan and SIM (single ion monitoring) modes were tested with the saponin Bb standard and one pea sample, and SIM mode was chosen as most sensitive method.

c) SIM method

In SIM mode, the four ions m/z 943.2 (aiming at saponin Bb $[M+H]^+$), m/z 965.2 (aiming at saponin Bb $[M+Na]^+$), 1069.1 (aiming at saponin βg $[M+H]^+$), and 1091.2 (aiming at βg $[M+Na]^+$) were selected, and for all of them the dwell time was selected to be 100 seconds. No fragmentor energy was used. The sodium adducts had the highest peak intensities and were therefore used for semi-quantification (Table 4).

Table 4 Results of the saponin analyses

Retention time (min)[a]	Compound	Monoisotopic mass	Detected ion $[M+H]^+$	Detected ion $[M+Na]^+$
44.8	saponin Bb ("saponin B")	942.2	943.2	965.2
50.2	saponin βg ("DDMP saponin")	1070.2	1069.1	1091.2

[a] slightly variable when new mobile phases are prepared

d) Calculation of corrected relative peak areas

The peak areas of the saponin peaks were corrected for the net weight differences in order to correct for small differences during weighing of 200mg pea flour, and to correct for moisture content of the different storage conditions. Moisture content was measured again just before LC-MS analysis (data not shown).

3 Results

3.1 Pea weight

The pea weight change during storage is given in Figure 4 (referred to as ' Fresh weight change'). The largest changes occurred during the first week of storage. Within each temperature, the high RH resulted in an increase of pea weight while the low and middle RH resulted in a decrease of pea weight. Storage at 40 °C resulted in more weight loss than storage at 12 °C.

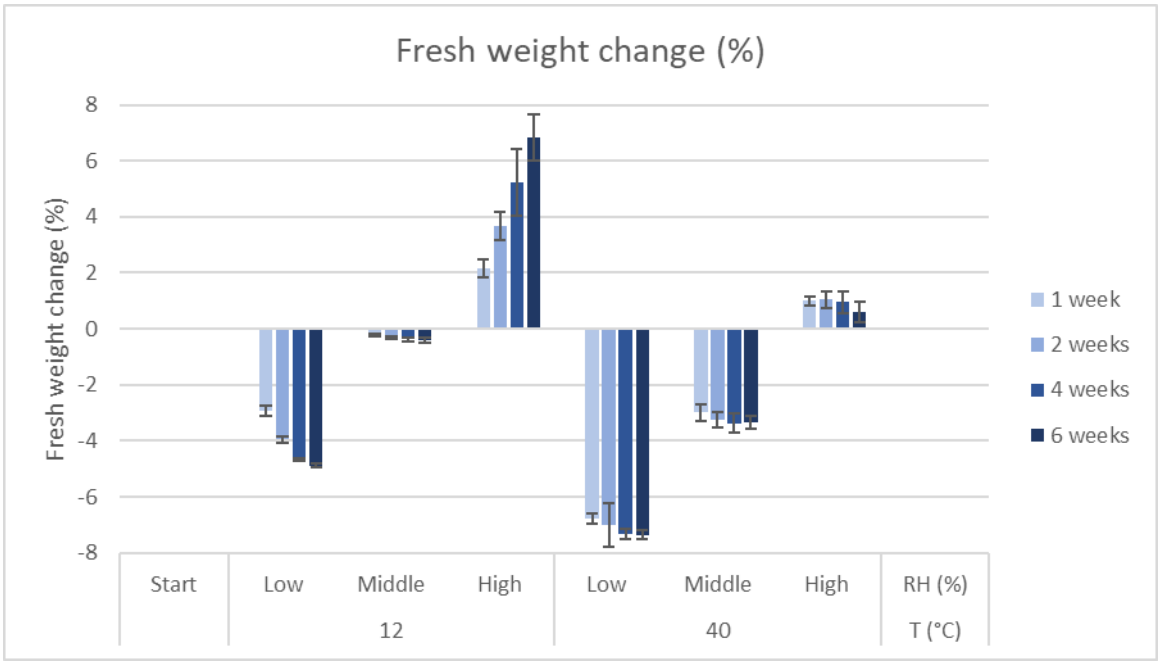


Figure 4 ***Weight change of peas stored at different conditions during various storage periods. Data are means +/- 95% CI, n =4.***

3.2 Moisture content

The moisture content of the starting material was 12.4% (Figure 5). The middle RH but especially the low RH led to a decrease in moisture content. The lowest value (4.8%) was reached after 6 weeks storage at low humidity and 40 °C. The high RH resulted in an increase of moisture content at 12 °C (up to 17.6%) while there was no clear change at 40 °C. Storage at 40 °C resulted in a lower moisture content than storage at 12 °C.

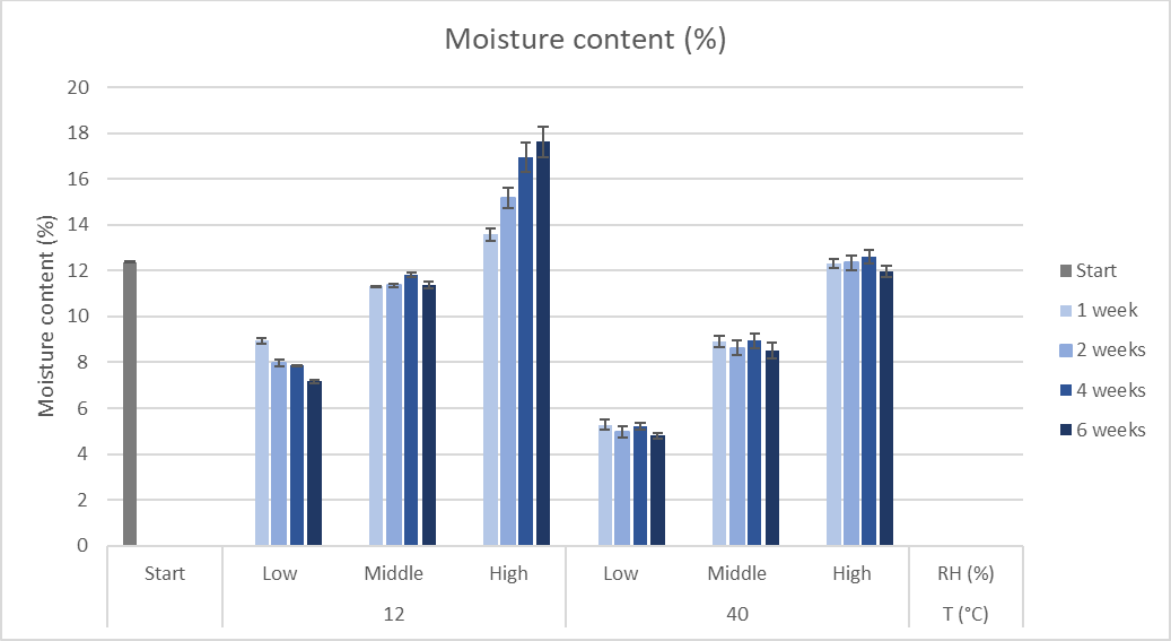


Figure 5 ***Moisture content (%) of peas stored at different conditions during various storage periods. Data are means +/- 95% CI, n =4.***

3.3 Colour

The colour varied between individual peas within samples, also for the starting material (Figure 6). There were no clear differences between samples, anyway not enough to be regarded as practically relevant.

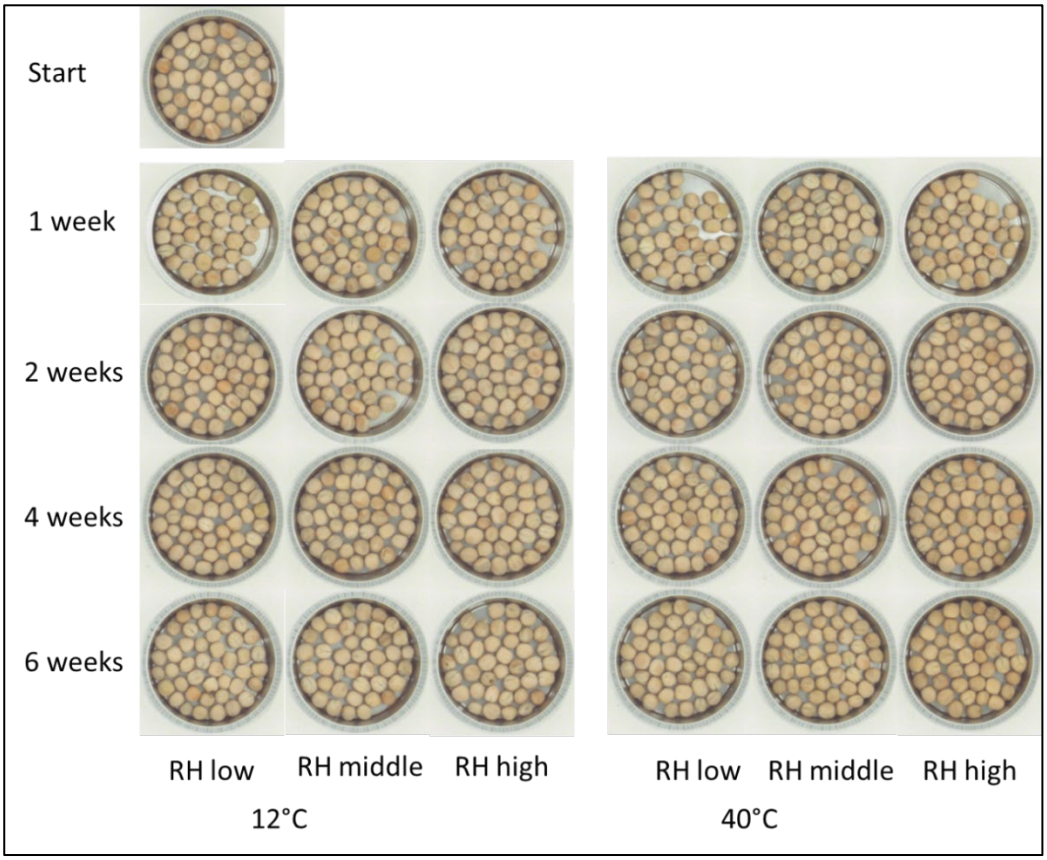


Figure 6 ***Corrected images of the peas stored at different conditions during various storage periods.***

3.4 Protein content (BCA assay)

The protein content was quantified for the starting material and the 24 combinations of temperature, RH and storage period. The results of the BCA assay generally showed a wide variation in protein content between the two replicates of most treatments (Fig 7, CI error bars). This was also the case for the starting material. The starting material contained 203 mg BSA/g FW. This was equivalent to 20,3% of protein on fresh basis, and 23.2 % on dry basis. Overall, there was no clear effect of the storage condition (temperature, RH) and the storage period on protein content.

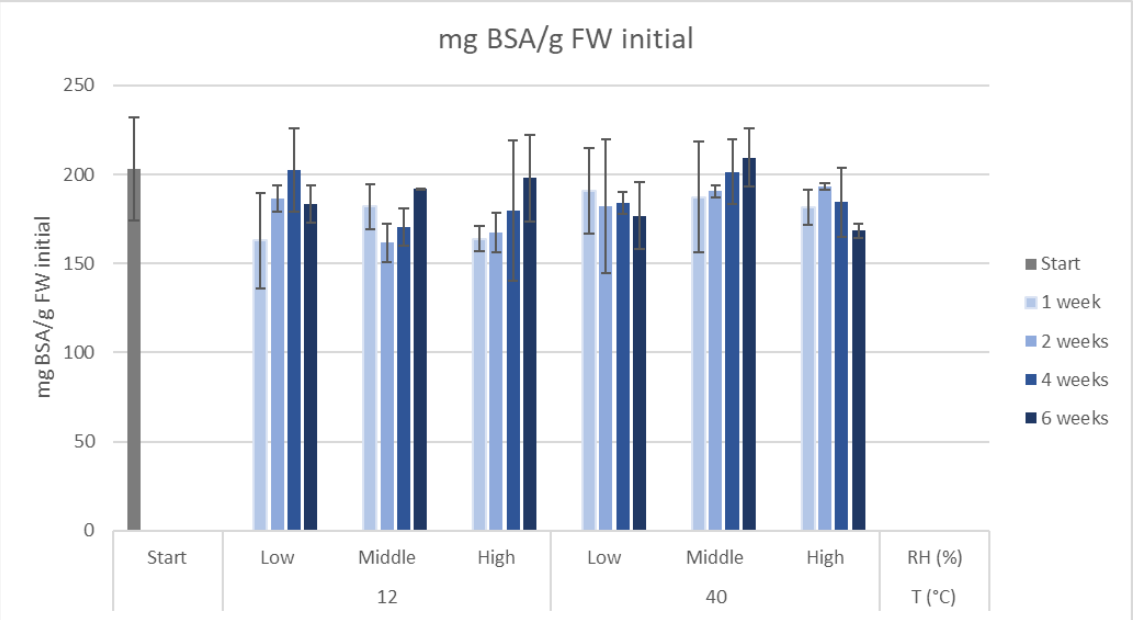
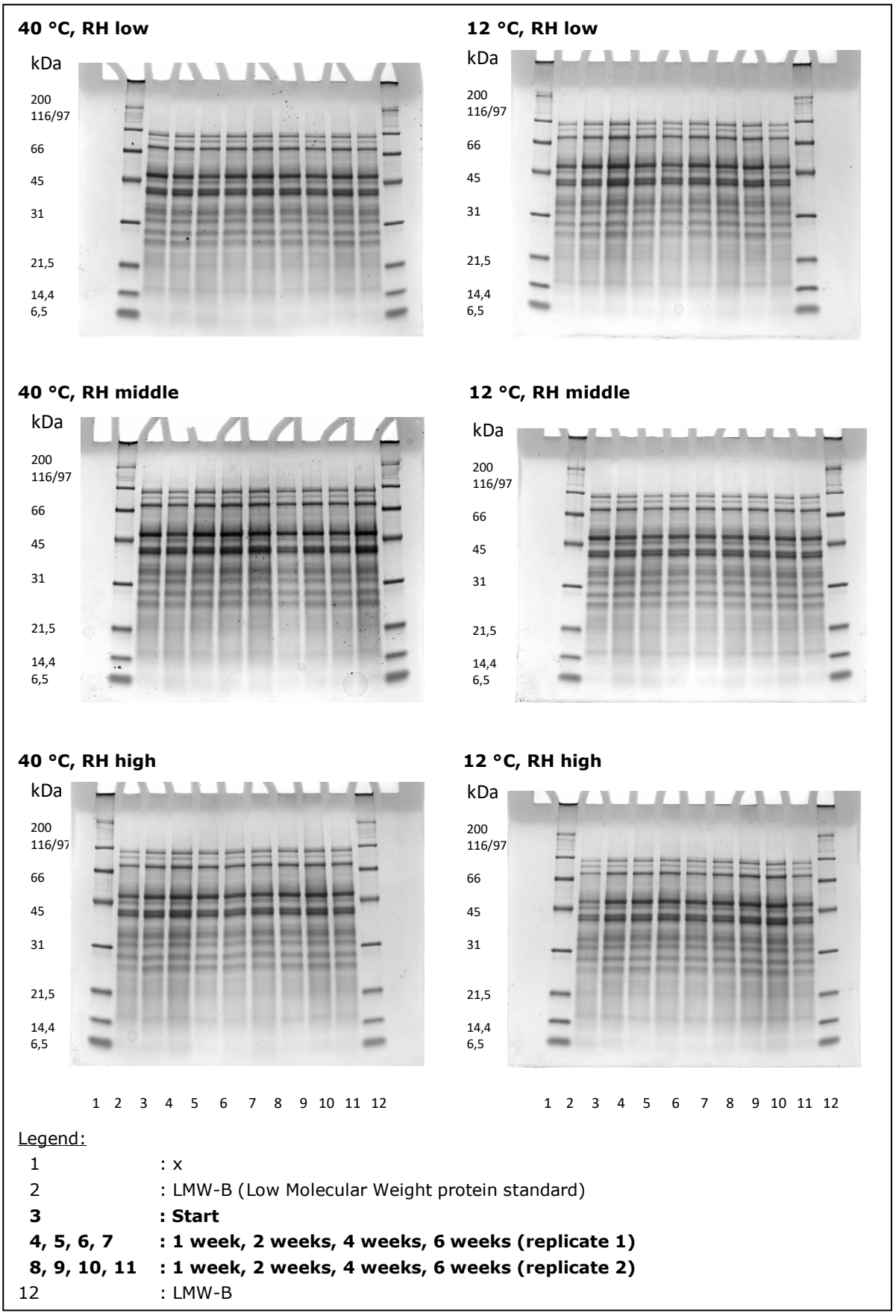


Figure 7 ***Protein content of peas stored at different conditions during various storage periods. Protein content is expressed in mg protein per g initial fresh weight (= at start of the trial). Data are means +/- 95% CI, n = 2.***

3.5 Protein composition (SDS-page)

SDS-page was used to compare the protein composition for the starting material and the 24 combinations of temperature, RH and storage period. Comparison between the bands on the protein gels showed no effect of storage conditions on extracted proteins (Figure 8). There was no significant protein aggregation (which would have been visible in the top of the gels).



Legend:

1	: x
2	: LMW-B (Low Molecular Weight protein standard)
3	**: Start**
4, 5, 6, 7	**: 1 week, 2 weeks, 4 weeks, 6 weeks (replicate 1)**
8, 9, 10, 11	**: 1 week, 2 weeks, 4 weeks, 6 weeks (replicate 2)**
12	: LMW-B

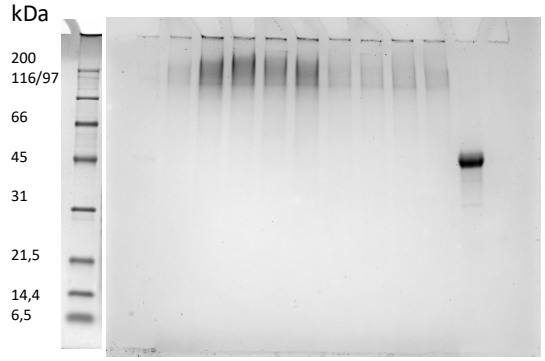
Figure 8 SDS page gels of proteins extracted from peas stored at different conditions during various storage periods.

3.6 Glycation

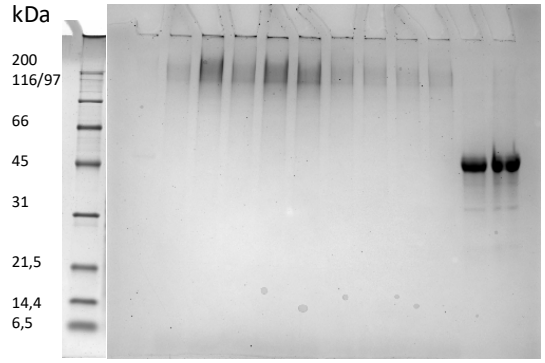
3.6.1 Glycation 1st experiment

A small subset of samples was analysed to get a first impression of the relevance of storage conditions for glycation. The gels clearly indicated the presence of glycoproteins (Figure 9). The most prominent bands were found after storage at low RH, at both temperatures (lines 3-6). The results indicate that the glycation had clearly increased since the start of storage (line 2), and had already increased after 1 week of storage (line 3). Bands after storage at high RH (lines 7-10) were similar to the starting material, indicating that no glycation took place during storage.

12 °C, low and high RH



40 °C, low and high RH



1 2 3 4 5 6 7 8 9 10 11 12

Legend:

1	: LMW-B (Low Molecular Weight protein standard)
2	**: Start**
3, 4, 5, 6	**: Low RH; 1 week, 2 weeks, 4 weeks, 6 weeks**
7, 8, 9, 10	**: High RH; 1 week, 2 weeks, 4 weeks, 6 weeks**
11	: HRP (positive control)
12	: HRP

Figure 9 SDS page gels for glycoprotein detection, extracted from peas stored at different conditions during various storage periods.

3.6.2 Glycation 2nd experiment

Based on the results of the first test, the question was whether glycoproteins are related to the (dehydrated) hull. In this case, the glycation would not/less occur in the cotyledons after dehulling. Therefore, a small set of samples was used to test whether glycation would still be found for the cotyledons, after dehulling.

The BCA assay showed a similar protein content between the extracts of pea with and without hull (Figure 10). This was followed by a SDS-page for protein composition (Figure 11) and a SDS-page with PAS staining for glycoproteins (Figure 12).

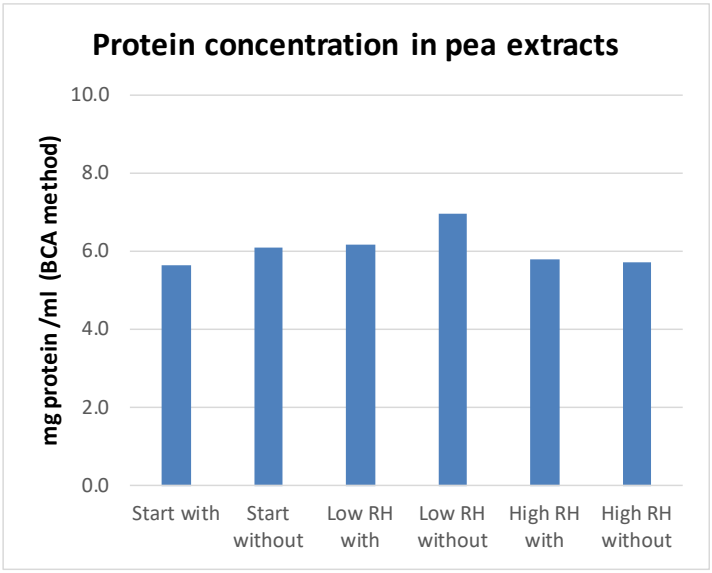
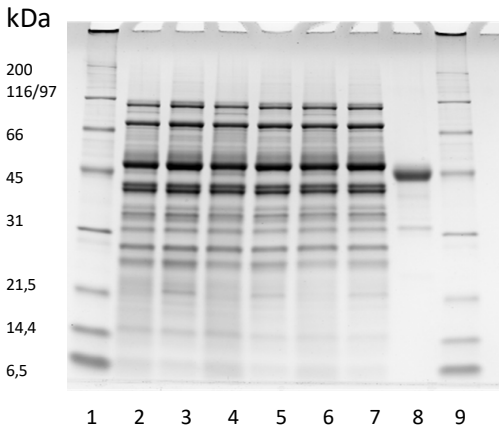


Figure 10 ***Protein concentration in extracts of pea with and without hull, of starting material and of peas stored during 1 week at low and high RH.***

Starting material and 1 week 12 °C (low and high RH)

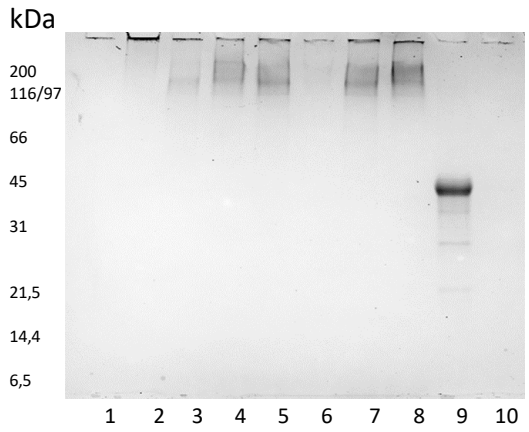


Legend for gel:

1	: LMW-B
2	**: Starting material with hull**
3	**: Starting material without hull**
4	**: Low RH, 1 week, with hull**
5	**: Low RH, 1 week, without hull**
6	**: High RH, 1 week, with hull**
7	**: High RH, 1 week, without hull**
8	: HRP
9	: LMW-B

Figure 11 ***SDS page gel of proteins after extraction of soluble proteins, from peas of starting material and after storage during 1 week at low and high RH, and then partly dehulled.***

Starting material and 1 week 12 °C (low and high RH)



Legend:

1	: x
2	: LMW-B
3	**: Starting material with hull**
4	**: Starting material without hull**
5	**: Low RH, 1 week, with hull**
6	**: Low RH, 1 week, without hull**
7	**: High RH, 1 week, with hull**
8	**: High RH, 1 week, without hull**
9	: HRP
10	: LMW-B

Figure 12 SDS page gels for glycoprotein detection, extracted from peas stored at different conditions during various storage periods, and then partly dehulled.

With the exception of the starting material, the 2nd glycation experiment was carried out with the same pea sample (the same replicate) as in the 1st experiment. Therefore, the following comparisons can be made:

- Line 3 of the 1st experiment (Figure 9, upper gel 12 °C) and line 5 of the 2nd experiment (Figure 12). Both show a similarly clear formation of glycoproteins.
- Line 7 of the 1st experiment (Figure 9, upper gel 12 °C) and line 7 of the 2nd experiment (Figure 12). The 2nd experiment shows a clearer formation of glycoproteins.

This 2nd experiment shows a clear glycation after storage at high RH. Comparison of samples with and without hull is confusing: The glycation is more for samples with hull of the starting material and of samples of high RH. While it is the opposite for samples of low RH.

In general, this 2nd experiment confirmed that differences in glycation occur between samples. While the explanation behind these differences is not clear, it is an interesting finding that glycation seems to be influenced. However, the different finding between the 1st and 2nd experiment cannot be explained. This made a further experiment worthwhile.

3.6.3 Glycation 3rd experiment

Based on the results of the previous experiment, it was decided to carry out analyses in addition to standard plant protein extraction using an reduced extraction buffer for total protein. Most analyses were carried out with newly processed samples. Also, some analyses were repeated with samples (plant protein-extracted) that were still available from the 2nd experiment to test whether the results are reproducible.

The BCA assay of total proteins again showed a similar protein content in the extracts of pea with and without hull (Figure 13). This was followed by a SDS-page for protein composition (Figure 14). Comparison between the bands on these protein gels showed no effect of storage conditions on proteins after extraction of plant proteins.

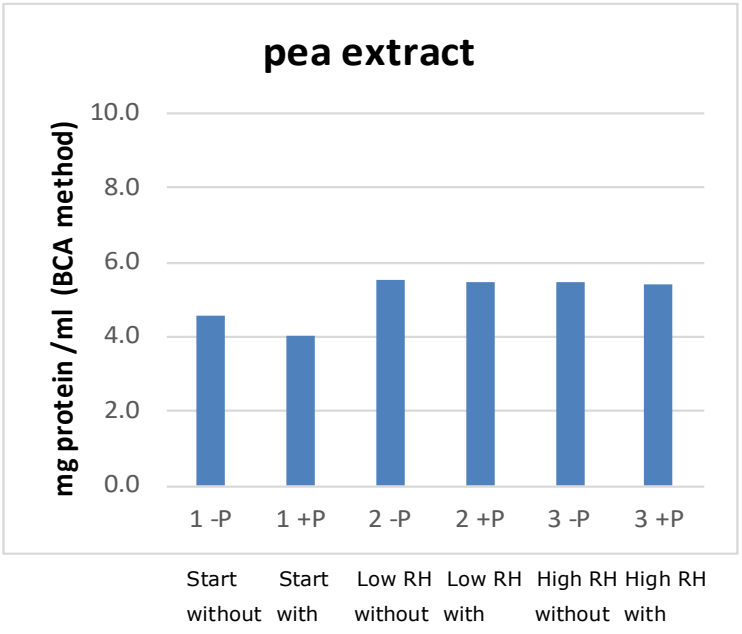
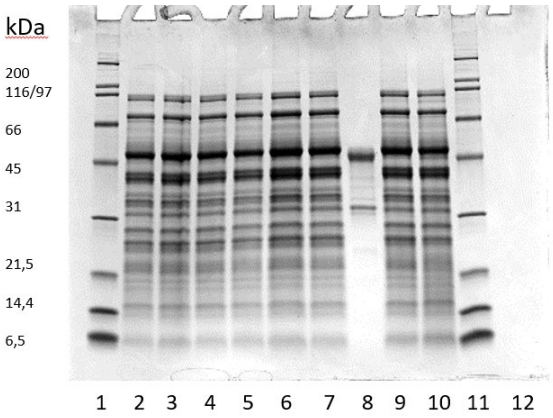


Figure 13 ***Protein concentration in extracts of pea with and without hull, of starting material (1) and of peas stored during 1 week at low RH (2) and high RH (3).***

Starting material and 1 week 12 °C (low and high RH)



Legend for gel:

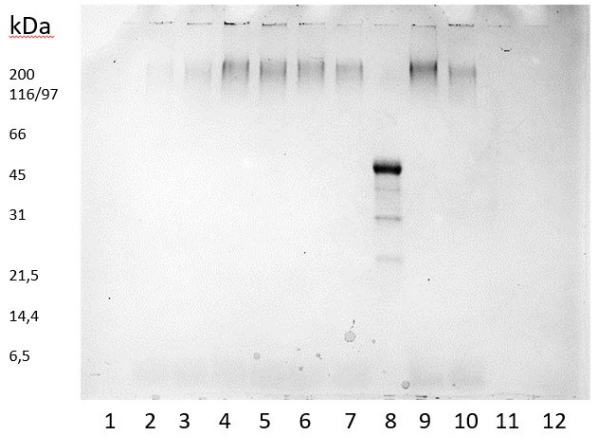
1	: LMW-B
2	**: Starting material without hull**
3	**: Starting material with hull**
4	**: Low RH, 1 week, without hull**
5	**: Low RH, 1 week, with hull**
6	**: High RH, 1 week, without hull**
7	**: High RH, 1 week, with hull**
8	: HRP
9	: **Starting material without hull** (same sample as 2nd experiment)
10	: **Starting material with hull** (same sample as 2nd experiment)
11	: LMW-B

Figure 14 ***SDS page gel of proteins after extraction of soluble proteins, from peas stored at different conditions during various storage periods, and subsequently partly dehulled.***

Figure 15 shows the SDS-page for glycoproteins detection, after extraction of soluble plant proteins. The newly processed samples (lines 2 to 7) indicated that glycation occurred between the start of storage and 1 week storage. This was not affected by RH level or by dehulling.

The repeated analyses of two samples of the 1st experiment confirmed the previous results: Lines 9 and 10 (Figure 15) were in accordance with lines 4 and 3 respectively (Figure 12).

Starting material and 1 week 12 °C (low and high RH)



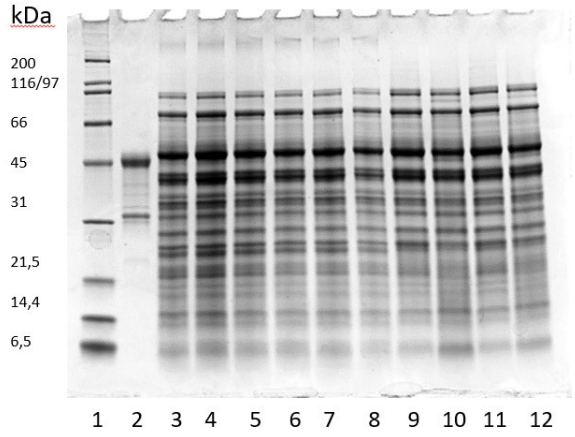
Legend:

1	: LMW-B
2	**: Starting material without hull**
3	**: Starting material with hull**
4	**: Low RH, 1 week, without hull**
5	**: Low RH, 1 week, with hull**
6	**: High RH, 1 week, without hull**
7	**: High RH, 1 week, with hull**
8	: HRP
9	: **Starting material without hull** (same sample as 2nd experiment)
10	: **Starting material with hull** (same sample as 2nd experiment)
11	: LMW-B

Figure 15 ***SDS page gels for glycoprotein detection, after extraction of soluble plant proteins, from peas stored at different conditions during various storage periods, and then partly dehulled.***

Also after extraction of total proteins under reduced conditions, the SDS-page showed no effect of storage conditions on protein composition (Figure 16, lines 3-8).

Starting material and 1 week 12 °C (low and high RH)



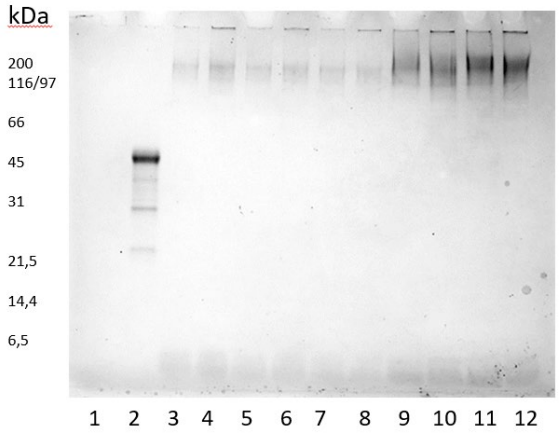
Legend for gel:

1	: LMW-B
2	: HRP pos. control
3	**: Starting material without hull**
4	**: Starting material with hull**
5	**: Low RH, 1 week, without hull**
6	**: Low RH, 1 week, with hull**
7	**: High RH, 1 week, without hull**
8	**: High RH, 1 week, with hull**
9	**: Low RH, 1 week, without hull** (same sample as 2nd experiment)
10	**: Low RH, 1 week, with hull** (same sample as 2nd experiment)
11	**: High RH, 1 week, without hull** (same sample as 2nd experiment)
12	**: High RH, 1 week, with hull** (same sample as 2nd experiment)

Figure 16 ***SDS page gel of proteins after extraction of total proteins (lines 3-8) and after extraction of soluble plant proteins (lines 9-12). Extractions are from peas stored at different conditions during various storage periods, and subsequently partly dehulled.***

Figure 17 shows the SDS-page with PAS staining for glycoproteins after extraction of total proteins (lines 3-8). These newly processed samples (lines 3 to 8) indicated that no glycation occurred between the start of storage and 1 week storage. This is in contrast to our findings after extraction of soluble proteins. Again, the glycation was not clearly influenced by RH level or by dehulling.
The repeated analyses of four samples of the 1st experiment were not in line with the previous results. The previously found significantly lower glycation for 'Low RH, 1 week, without hull' (Figure 12, line 6) could not be confirmed (Figure 17, line 9).

Starting material and 1 week 12 °C (low and high RH)



Legend for gel:

1	: LMW-B
2	: HRP pos. control
3	**: Starting material without hull**
4	**: Starting material with hull**
5	**: Low RH, 1 week, without hull**
6	**: Low RH, 1 week, with hull**
7	**: High RH, 1 week, without hull**
8	**: High RH, 1 week, with hull**
9	**: Low RH, 1 week, without hull** (same sample as 2nd experiment)
10	**: Low RH, 1 week, with hull** (same sample as 2nd experiment)
11	**: High RH, 1 week, without hull** (same sample as 2nd experiment)
12	**: High RH, 1 week, with hull** (same sample as 2nd experiment)

Figure 17 ***SDS page gels for glycoprotein detection, after extraction of total proteins (lines 3-8) and after extraction of soluble plant proteins (lines 9-12). Extractions are from peas stored at different conditions during various storage periods, and then partly dehulled.***

3.6.4 Glycation 4th experiment: Differential Scanning Calorimetry (DSC)

An example of a DSC thermogram is given in Figure 18. Enthalpy was calculated from the area of the transition peak. The results for the 6 samples are given in Table 5.
A single endotherm peak around 84°C was observed for all 6 samples, suggesting that the protein liquids consisted of species with similar thermostability. The difference in denaturation temperature between samples with and without hull is very small and may be explained by a difference in protein composition.
When comparing these results with the degree of glycation in the former experiments, there is no clear relationship between glycation and thermal properties of the pea proteins.

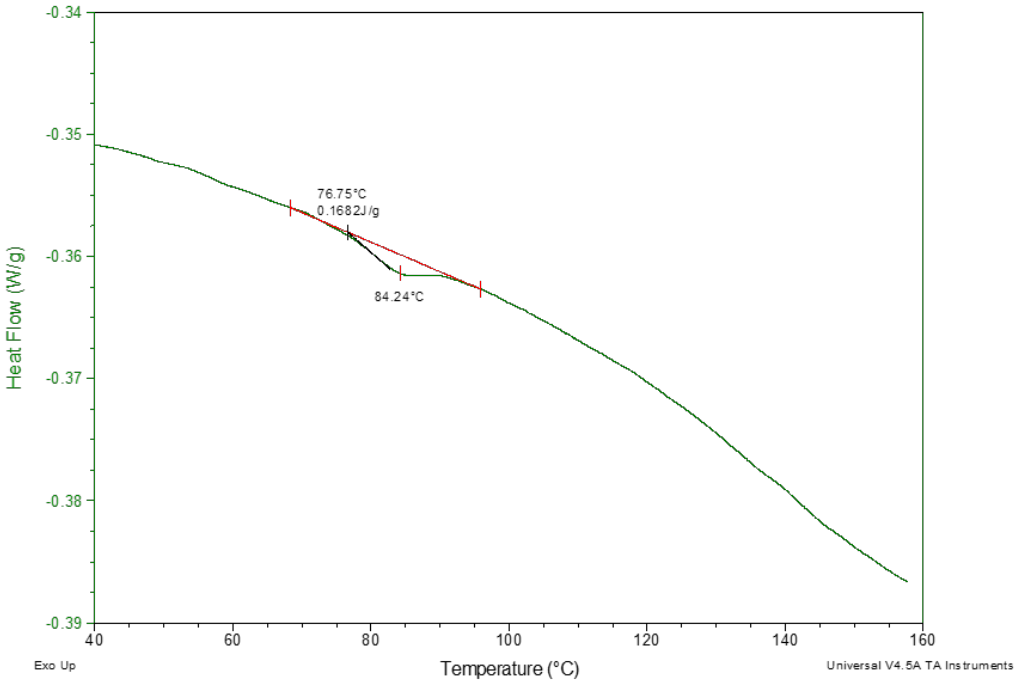


Figure 18 ***Example of DSC thermogram with T onset and denaturation temperature Td. (sample: starting material with hull).***

Table 5 ***Results of DSC for the 6 pea flour samples.***

Treatment	T onset	Td peak	Enthalpy (J/g)	
			on total weight	corrected for MC
Starting material without hull	77.91	84.77	0.08	1.50
Starting material with hull	76.75	84.24	0.17	3.33
Low RH, 1 week, without hull	78.86	84.69	0.11	2.12
Low RH, 1 week, with hull	77.27	84.21	0.18	3.60
High RH, 1 week, without hull	77.14	85.41	0.09	1.73
High RH, 1 week, with hull	76.73	84.66	0.21	4.17

3.7 Hexanal

The results of the hexanal analyses are expressed in absolute peak area (Table 6). The absolute areas of samples determined in dry condition are not comparable with those from the wet method because of the difference in flavour release from the matrix. Only within one method (dry or wet) the samples can be compared. Twice as much area means that the hexanal concentration in the headspace is double.

Table 6 ***Moisture content (%) of pea samples used, and corresponding hexanal (area) measured in the headspace above dry (milled) peas and above milled peas in a 50% watery solution. (hexanal peak area not corrected for moisture content). For each treatment, data of two replicates are given.***

Storage conditions			Dry method results		Wet method results	
Storage period	Temperature	RH	Moisture content (%)	Hexanal peak area	Moisture content (%)	Hexanal Peak area
Start	-	-	12,4	40	12,4	61
			12,3	203	12,3	110
6 weeks storage	**12 °C**	low	7,1	683	7,1	159
			7,2	811	7,3	155
		high	17,2	12	16,9	150
			18,3	8	18,1	152
	40°C	low	4,6	1055	4,7	103
			4,9	1391	4,9	178
		high	12,2	72		-
			11,8	93	11,8	141

When looking at the dry method results, the storage at high humidity led to clearly less hexanal in the headspace compared to storage at low humidity. As hexanal is a marker compound for oxidation, while other oxidation products have more influence on off-flavour, this finding indicates that less oxidation odour might occur after storage at higher humidity. It looks like the samples stored at low humidity are "flavour based" (thus of an inferior quality). However, when looking at wet method results, no differences are seen in level of oxidation (Table 4 last column). While differences between the storage temperatures were found for the dry method, this was also not the case anymore for the wet pea material.

3.8 Saponins

The HPLC-MS analyses were performed for starting material (week 0), and for samples kept at 12 and 40 °C after 6 weeks storage at 25 and 85% RH (target RH). For each condition, two technical duplicates (extracts made from peas from two duplicate storage containers) and two analytical duplicates (extracts measured twice) were analysed on HPLC-MS. The results are depicted in Figure 19. Soyasaponin βg ("DDMP saponin"), is found at higher levels in the starting material than soyasaponin Bb ("saponin B"). This was expected, as saponin βg is the native saponin in peas (Heng et al., 2006). After 6 weeks of storage, the saponin βg content for peas kept at 12°C at 25 and 85% RH, seems not to have changed significantly (only a trend of a very slight decrease). The same holds for 40°C at 25% RH. However at 40°C at 85% RH it can be observed that saponin Bb content

increased, whereas the saponin βg content decreased, with a total saponin content at the same level as under all the other conditions. It could thus be that saponin βg was partially degraded to saponin Bb under the combination of "more extreme" conditions of 40°C and 85% RH. However, this result might also be explained by the fact that the peas of this particular condition (both duplicate containers) were ground on another day than the peas of all the other conditions. Despite the fact, that at both grinding times, liquid nitrogen was applied in order to prevent heat development, and grinding times were short (a few seconds), it might be possible that the observed results are a consequence of sample preparation rather than an effect of storage conditions. In general, it can be said that the expected impact on bitter taste and astringent mouthfeel is approximately the same under all conditions analysed by HPLC-MS. In case that the higher saponin Bb and lower saponin βg content of the 40°C / 85% RH samples was a real storage effect, the taste of these samples might be slightly better than of all the other samples, as saponin Bb is slightly less bitter than saponin βg. But in general, the tested storage conditions did not seem to have a large impact on the bitter taste of the peas.

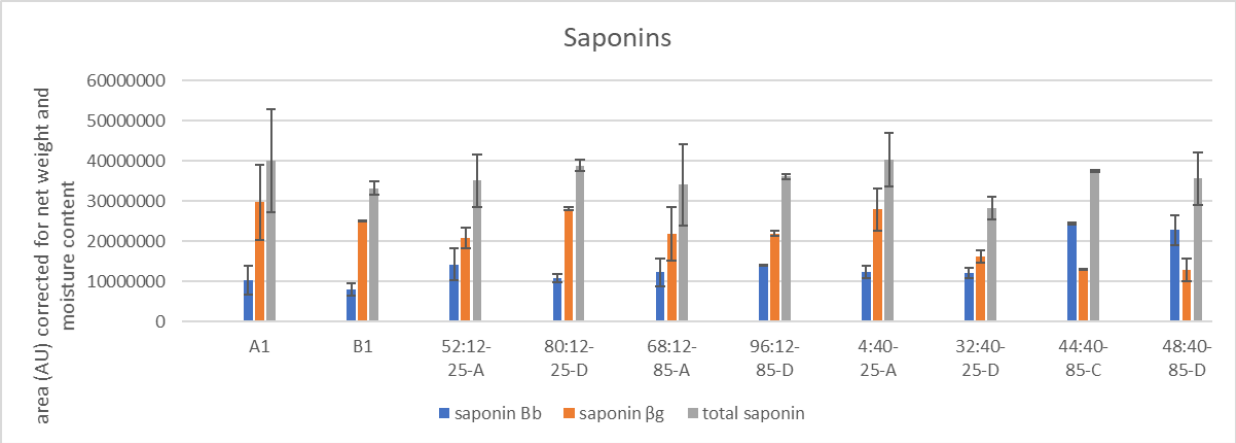


Figure 19 Relative contents of saponins in peas under starting conditions (A1, B1), and after 6 weeks kept at 12°C and 25%RH (52:12-25-A, 80:12-25-D), 12°C and 85%RH (68:12-85-A, 96:12-85-D), 40°C and 25%RH (4:40-25-A, 32:40-25-D), and 40°C and 85%RH (44:40-85-C, 48:40-85-D).

4 Discussion and Conclusion

4.1 Pea weight, moisture content, and colour images

The observed changes in pea weight ('fresh weight') (%) correspond to the changes in moisture content (%). The moisture content of the starting material was 12.4%, which is on the low side within the range for storage in practice (Table 1). Further storage at low RH led to a further decrease to a very low moisture content, to 5.3% already after 1 week of storage at 40 °C, and to 4.8% after 6 weeks. These values are much lower than common in practice. The highest moisture content was reached after storage at 12 °C for 6 weeks namely 17.6%. This does not seem to be an exceptional value. Peas are considered dry at moisture content < 16%, tough at 16.1-18% and damp above 18% (Barker, 2018).

The South Dakota State University has recently published a study about storage of different pulses including yellow peas (Hall et al., 2020). Storage temperatures were 22, 40 and 50 °C; RHs were between 40 and 85%; storage periods were up to 90 days. Changes in moisture content over time showed similar trends to those in our present study.

The (colour) images confirmed that no germination or other visual disturbing changes had occurred. The realized differences in moisture content in this experiment provided a good basis for our goal to study the effect on protein content, composition and functionality.

4.2 Protein content and composition

Overall, there was no clear effect of storage conditions (temperature, RH) and storage period on the protein content, as shown by the BCA assays. A critical note can be the large variation in protein content between the two replicates of the starting material and between the two replicates of each treatment. Perhaps the protein content varies widely between individual peas. The storage conditions and storage period also did not affect protein quality in terms of protein composition, as shown by SDS pages. Also Hall et al. (2020) found only minor changes in protein % during 90 days storage.

These results are beneficial for practical storage. It should be noted that the peas had been stored already for several months before the experiment started and that the results are only valid for these tested circumstances.

Hall et al. (2020) have looked at many aspects regarding the impact of storage conditions on the functionality of yellow pea. Slight downward trends over time were observed in foaming capacity and stability, and the researchers concluded that this supports a possible change in protein structure or composition. Among other things, they also observed downward trends with increasing RH and storage temperature in pasting temperature, gel firmness, and cold past viscosity.

4.3 Glycation

A very interesting part of our research was the study on glycation. Glycation of proteins is able to improve their techno-functional properties. But the so-called glycoconjugates resulting from glycation are not available as commercial ingredients for food applications (Kutzli et al., 2020). This is because methods are not easy to scale-up. Furthermore, the established dry state method involves expensive freeze-drying, and is not easily controllable in terms of unwanted reaction products. Also, the conjugation of sugar moiety to protein is a process occurring during storage of powders, but the process should not take place on purpose. This process will be considered as novel foods. Therefore, we speculate that a storage method which increases glycation can have commercial value.

To our knowledge no study has investigated before to what extent the storage conditions of yellow pea affect glycation. Our described 1st experiment on glycation indicated that glycation occurred after storage in low humidity, already after 1 week. This may be related to the very low applied RH and consequently low moisture content, which also occurred already after 1 week. However, our following experiment could not confirm this effect of low humidity. Moreover, some contradictory results were found between the consecutive experiments.

This higher level of glycation under dryer conditions, as found in the 1st experiment, has similarities with the following findings:

- For *Arabidopsis thaliana*, a higher level of protein glycation was found in relation to osmotic stress during field conditions (Paudel et al., 2016).
- Leonova et al. (2020) suggested that short-term drought of pea plants on the field might result in enhancement of protein glycation, and in accumulation of the resulting advanced glycation end products (AGEs) in filling pea seeds. They expect that drought stress might result in a pronounced decrease in protein and lipid contents, accompanied with increase in sugar contents (referring to (Nakagawa et al., 2018)). As was shown for Arabidopsis leaf, these changes can be accompanied with increase of glycation levels (Paudel et al., 2016; Chaplin et al., 2019). However, for mature seeds this aspect is still to be addressed in future studies (Leonova et al., 2020).
- In plants, water deficit ultimately results in the development of oxidative stress and accumulation of osmolytes (e.g. amino acids and carbohydrates) in all tissues (Paudel et al., 2016). Up-regulation of sugar biosynthesis in parallel to the increasing overproduction of reactive oxygen species (ROS) might enhance protein glycation and advanced AGEs.

Saldanha do Carmo et al. (2020) found that dehulling had no impact on the techno-functional properties of the produced fractions apart from a slight improvement of the emulsifying capacity of pea fine fractions.

Overall, in our study, we found differences in glycation between samples and storage conditions. Although the explanation behind these differences is not clear, it is an interesting finding that glycation can be influenced. This makes further research worthwhile.

4.4 Hexanal and saponins

There was no evidence of an effect of storage on off-flavour compounds. Both hexanal and saponins are interesting compounds in relation to off-flavour in peas (Roland et al., 2017).

In our experiment, the 'dry' method for hexanal analyses, in the headspace above the samples, showed clear differences between the treatments. It may seem that, based on smelling of dry peas, the samples with low moisture content were of inferior flavour quality (higher hexanal in the headspace). However, this observation can be attributed to the fact that hexanal is more easily released from peas with a low moisture content. The analyses with the 'wet' method indeed did not show differences between the treatments. The latter is of practical relevance for processed products.

The saponins analyses revealed that both saponins generally expected in peas, soyasaponin βg ("DDMP saponin") and soyasaponin Bb ("saponin B") were present in the starting material and under all storage conditions. Under most conditions, the content of the slightly more bitter native saponin βg was higher than the content of the slightly less bitter degradation product saponin Bb. At 40°C / 85% RH, the result was βg < Bb, which might be either a real effect, or might be caused by the milling step of these samples during sample preparation. In case of a real effect, it can be expected that peas kept at higher temperature in combination with higher relative humidity might have a slightly reduced bitterness. However the differences are small and in conclusion it can be said that the tested storage conditions do not seem to have a large impact on the saponin profile and therewith on (part of) the bitter taste of the peas.

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