MASTERARBEIT | MASTER'S THESIS

Titel | Title

Comparing the protein profile of fermented and roasted soybeans by high resolution LC-MS

verfasst von | submitted by

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angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien | Vienna, 2025

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 066 862
Studienrichtung lt. Studienblatt | Degree programme as it appears on the student record sheet:	Masterstudium Chemie
Betreut von | Supervisor:	Ass.-Prof. Dipl.-Ing. Evelyn Rampler Privatdoz. Bakk. PhD

Preface

The work of the present master's thesis was a cooperation between the University of Vienna and the Austrian Agency for Health and Food Safety (AGES). The work was conducted from July 2024 to December 2024 at the Department for Feed Analysis and Quality Testing, Institute for Animal Nutrition and Feed, AGES under the supervision of Dr. Stefano D`Amico and Dr. Elisabeth Reiter as well as Ass.-Prof. Dipl.-Ing. Evelyn Rampler from the Department of Analytical Chemistry, Faculty of Chemistry, University of Vienna.

Acknowledgements

My sincerest gratitude to my lab supervisor Sara Schlachter for her constant and warming presence throughout the six months I had the honours of spending at the Austrian Agency for Health and Food Safety (AGES). Her previous efforts have laid the groundwork for my part in this project, allowing me to have the best possible starting point, and for that I am eternally grateful. I deeply appreciate the chance I was given by Dr. Elisabeth Reiter and Dr. Stefano D`Amico to become part of the team and develop under the experts at the Department for Feed Analysis and Quality Testing. Their support nurtured my abilities and gave me the confidence to thrive in a professional environment. Moreover, I want to express just how wonderful having Ass.-Prof. Dipl.-Ing. Evelyn Rampler as my supervisor has been and how very thankful I am for her support. Her undying reassurance and never-ending patience were just as valuable to me as her incredibly insightful academic input, which made the finishing of this thesis even possible. Last but not least, my endless love and admiration to my parents who have tirelessly provided me with the means and encouragement to go after my dreams and achieve my goals. Their belief in me got me through any hardship during my years away from home, and I could not have wished for more.

Abstract

Soybeans are a convenient source of high-quality protein with a mostly well-balanced amino acid profile, making them a viable feed option. However, the presence of intrinsic anti-nutritional factors (ANFs) reduces their nutritional value, especially trypsin inhibitors (TIs). Thus, soybean needs to be processed before administering it as feed. While roasting can reduce ANFs and make soybean more digestible, it can cause loss of nutrients due to the occurrence of Maillard reactions. Fermentation can be used as an alternative processing technique as it has also been shown to effectively reduce ANFs and improve digestibility. In this work, four different soybean varieties (Atacama, Lenka, GL Melanie and ES Mentor) were subjected to a submerged fermentation method using a mixture of silage additives consisting of lactic acid bacteria strains and compared to samples roasted at 225 °C for 290 seconds. Marginal difference was recorded in the crude protein content between raw and roasted samples, which were around 39-41 % on a dry matter basis, while the fermented samples afforded values of 32-35 %. The soluble protein content in the raw samples revealed values around 22-24 % on dry matter basis, whereas roasted samples showed lower content around 18-17 % and the fermented samples between 21-18 % on dry matter basis. No notable change was observed in the amino acid composition but in the amino acid content, which measured lower in the fermented samples. The relative change of the protein profiles was investigated using LC-MS, where selected proteins were analysed through an untargeted approach in data-independent acquisition (DIA) mode on a high-resolution quadrupole time-of-flight (QTOF) mass spectrometer. While the relative content of some proteins seemed unaffected by either roasting or fermentation, some showed a significant decrease after both. No stark difference was observed between both treatments themselves except for few cases where fermentation caused a slightly bigger loss than roasting. The most prominent difference was observed in the trypsin inhibitor activity (TIA); the initial TIA of the raw soybeans decreased from 20-29 mg/g to 8-14 mg/g after fermentation and below 4 mg/g after roasting. Although fermentation revealed some benefits, further optimization is needed to achieve a TIA below the suggested threshold of 4 mg/g for feed.

Zusammenfassung

Sojabohnen sind eine praktische Quelle für hochwertiges Protein mit einem überwiegend ausgewogenen Aminosäureprofil und stellen daher eine geeignete Futteroption dar. Allerdings verringert das Vorhandensein intrinsischer antinutritiver Faktoren (ANFs) ihren Nährwert, insbesondere Trypsininhibitoren (TIs). Daher ist eine Verarbeitung der Sojabohnen vor der Verfütterung erforderlich. Durch Rösten lassen sich ANFs zwar reduzieren und die Verdaulichkeit steigern, allerdings treten dabei infolge von Maillard-Reaktionen häufig Nährstoffverluste auf. Als alternative Methode bietet sich die Fermentation an, da sie ebenfalls eine wirksame Verringerung der ANFs bewirkt und gleichzeitig die Verdaulichkeit verbessert. In dieser Arbeit wurden vier verschiedene Sojabohnensorten (Atacama, Lenka, GL Melanie und ES Mentor) einer Submersfermentation mit einer Mischung aus Futtermittelzusatzstoffen (Silierhilsfmittel) aus Milchsäurebakterienstämmen unterzogen und mit Proben verglichen, die bei 225 °C für 290 Sekunden geröstet wurden. Die relative Veränderung ihrer Proteinprofile wurde mittels LC-MS untersucht, wobei ausgewählte Proteine in einem ungezielten Ansatz im data-independent acquisition-Modus auf einem hochauflösenden Quadrupol-Time-of-Flight (QTOF) Massenspektrometer analysiert wurden. Während der relative Gehalt einiger Proteine durch Rösten oder Fermentation unbeeinflusst zu sein schien, zeigte sich bei anderen eine signifikante Abnahme nach beiden Verarbeitungsprozessen. Zwischen den beiden Verfahren selbst wurde kein signifikanter Unterschied festgestellt, mit Ausnahme weniger Fälle, bei denen die Fermentation einen etwas stärkeren Verlust verursachte als das Rösten. Ein geringer Unterschied wurde im Gesamtproteingehalt zwischen rohen und gerösteten Proben festgestellt, die bei etwa 39-41 % auf Trockenmassebasis lagen, während die fermentierten Proben Werte von 32-35 % erreichten. Es wurde keine bemerkenswerte Veränderung in der Aminosäurezusammensetzung festgestellt, wohl aber in der Menge, die in den fermentierten Proben leicht niedriger war. Der lösliche Proteingehalt in den rohen Proben lag bei etwa 1,12-1,20 mg/mL, während die gerösteten Proben niedrigere Konzentrationen von 0,88-0,91 mg/mL und die fermentierten Proben Werte zwischen 0,77-0,84 mg/mL aufwiesen. Der deutlichste Unterschied zeigte sich in der Trypsininhibitoraktivität (TIA); die anfängliche TIA der rohen Sojabohnen sank durch Fermentation von 20-29 mg/g auf 8-14 mg/g und durch Rösten auf unter 4 mg/g. Obwohl durch Fermentation einige Vorteile erkennbar wurden, ist eine weitere Optimierung notwendig, um für die Fütterung gewünschte TIA von unter 4 mg/g zu erreichen.

Abbreviations

- AAC Amino acid content
- ACN Acetonitrile
- Ala Alanine
- Ambic Ammonium bicarbonate
- ANFs Anti-nutritional factors
- ANOVA ANalysis Of VAriance
- Arg Arginine
- Asp Aspartic Acid
- BBI Bowman-Birk inhibitor
- BSA Bovine serum albumin
- CPC Crude protein content
- Cys Cysteine
- DIA Data independent acquisition
- DMSO Dimethylsulfoxide
- DTT Dithiothreitol
- Glu Glutamic Acid
- Gly Glycine
- HCl Hydrochloric acid
- His Histidine
- HPLC High-performance liquid chromatography
- HSD Honestly Significant Difference
- IAA Iodacetamide
- Ile Isoleucine
- KTI Kunitz trypsin inhibitor
- L-BAPA N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride
- LC Liquid chromatography
- Leu Leucine
- Lys Lysine
- Met Methionine
- MS Mass spectrometry
- NaOH Sodium hydroxide
- NH_4^+ Ammonium
- NMR Nuclear magnetic resonance
- NOR Norleucine
- Phe Phenylalanine
- Pro Proline

- SD Standard deviation
- Ser Serine
- SONAR Sound Navigation and Ranging
- SPC Soluble protein content
- TFA Trifluoracetic acid
- Thr Threonine
- TI(s) Trypsin inhibitor(s)
- TIA Trypsin inhibitor activity
- Tyr Tyrosine
- Val Valine

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1 Introduction

1.1 Soybeans as feed

The soybean (*Glycine max*) is a globally significant crop known for its high protein content. It is often fed in the form of soybean meal, the material remaining after the extraction of oil.[1] According to the Austrian Descriptive Variety List maintained by the Federal Office for Food Safety, soybean seeds contain around 36-46 % protein on a dry matter basis, making them a very rich plant-based protein source.[2] One of the key advantages of soybean proteins is their similarity to those of animal origin in terms of digestibility and amino acid composition, which is well-balanced except for slightly lower levels of sulphur-containing amino acids.[3] Despite this minor limitation, soybean protein provides a fine balance of essential and non-essential amino acids.[4]

1.1.1 Protein profile

The major seed storage proteins of soybean – namely glycinin and β-conglycinin, each consisting of multiple subunits – make up approximately 80-90% of the total protein content.[5] The proportion of the subunits can vary among different soybean genotypes.[6] Less abundant proteins include the basic 7S globulins, β-amylase, cytochrome c, lectin, lipoxygenase, urease and enzyme inhibitors.[5,6] The most abundant amino acid in the soybeans is glutamic acid followed by aspartic acid, whilst among the essential amino acids, leucine has the highest concentration followed by lysine. The sulphur-containing amino acids methionine and cysteine are present in very low amounts.[4]

1.1.2 Anti-nutritional factors

The presence of naturally occurring anti-nutritional factors (ANFs) limits the inclusion of raw soybeans in feed.[7] They contain several different kinds of ANFs, of which the trypsin inhibitors (TIs) are one of the most relevant present in soybean, making up about six % of the total protein content.[7] TIs inhibit the activity of trypsin and chymotrypsin, essential enzymes involved in the digestion of dietary protein[1]. Moreover, they cause pancreatic enlargement and excessive secretion of digestive enzymes, which causes an endogenous loss of amino acids and ultimately leads to a depressed growth and development of the animal.[8] The most well-characterized TIs in soybeans are the Kunitz trypsin inhibitors (KTI) and the Bowman-Birk inhibitors (BBI).[7] With a molecular weight of around 20 kDa[9], the KTI has been observed to contribute to the total trypsin inhibitor activity by 60 %[10] or even up to 80 %[11], with its inhibitory activity in strict relation to its amount as it forms a 1:1 stoichiometric complex with the active

site of the enzyme.[7] While the KTI inhibits trypsin strongly, it is a weak inhibitor of chymotrypsin.[9] On the other hand, the BBI has a molecular weight of 8 kDa and inhibits both trypsin and chymotrypsin strongly.[9] It is a small and compact protein with a rigid structure, exhibiting thermal stability due to having seven disulfide bonds[10], whereas the KTI is considered more heat-labile in comparison due to only having two disulfide linkages[11]. However, DiPietro and Liener (1989) indicate that the assessment of the BBI stability may be more complex than previously assumed.[9] Nonetheless, both TIs can be effectively inactivated through appropriate processing techniques.[9] The inactivation of these ANFs is essential to enhance the nutritional quality of soybeans prior to their use in animal feed. [1]

1.2 Soybean Processing

Effort must be made to eliminate ANFs and increase the digestibility of soybean efficiently without compromising its overall quality. To ensure safe consumption, it is important to lower the trypsin inhibitor activity (TIA) – which measures the unit of trypsin inhibited per unit of sample[12] – below the recommended threshold of 4 mg/g for feed[13]. One widespread processing method is the application high-temperature treatments, e.g. in the form of roasting, hot-air drying or boiling, among others. Conventional heat-processing can inactivate TIs without the influencing the total protein content of the soybean.[3] It also decreases the solubility of proteins, which can be beneficial for ruminants in the form of rumen bypass proteins.[14] However, prolonged heat-treatment at higher temperatures in an attempt to further decrease the TIA comes with considerable setbacks. The exposure to high temperatures results in the denaturation of proteins, destruction of amino acids and formation of harmful Maillard products, thus greatly damaging the nutritional quality of soybean.[1,15,16] Another way to reduce ANFs in soybean is by fermentation, a biological process performed by microorganisms like bacteria or fungi. Fermentation has long been used throughout human history to preserve food and improve its digestibility.[1] This metabolic process not only breaks down complex molecules but also produces bioactive metabolites. Beneficial microorganisms can be delivered to the gut as probiotics, where they can contribute to the gastrointestinal microbial community.[1] As a result, the overall nutritional value of fermented soy is elevated compared to its unprocessed form. Many studies have explored different species and strains to find the best conditions and combinations for the effective biodegradation of harmful compounds in soy to make it more suitable as a feed ingredient.[1,4] Commonly used species in the case of fungal fermentation are *Aspergillus spp.* or *Rhizopus oligosporus*. They eliminate ANFs, break down carbohydrates and increase the number of easier to absorb small peptides – effectively reducing triggers for digestive issues and enhancing digestibility. While the relative protein content may increase, the amino acid profile remains mostly unchanged.[4] Very similar changes occur within soy during bacterial fermentation. Most prevalent bacterial species used in soy fermentation

include *Bacillus subtilis* and *Lactobacillus plantarum*. Besides degrading TIs and decreasing the protein size, the content of specific free amino acids can increase depending on the species or strain of bacteria. In fact, the type of microorganism plays a key role in determining the levels of various nutritional components in the fermented end product, as each microorganism has its own metabolic activity leading to a different output.[4] Additionally, factors such as temperature and moisture also influence the fermentation process. For instance, in the case of solid-state fermentation, the beans are kept in natural environmental conditions with minimal moisture content, whereas submerged fermentation takes place in a liquid medium, which allows for more precise control. The duration can range from a day to a week, the temperature from 25 to 37 °C.[1,17] The choice of fermentation method ultimately depends on the bacteria or fungi used.

1.3 Aim of this thesis

This thesis is aiming at comparing the relative changes in the protein profile of roasted and fermented soybeans using high-resolution LC-MS and to determine whether one method is superior to the other in preserving or eliminating specific proteins. Furthermore, aspects such as the crude protein content, soluble protein content, amino acid content and trypsin inhibitor activity are investigated to provide an idea of the differences between raw, roasted and fermented soybeans. Lastly, a linear correlation between the trypsin inhibitor amount and trypsin inhibitor activity is screened for to reveal any potential association.

2 Materials and Methods

2.1 Soybean Samples

The soybean varieties Atacama, Lenka, GL Melanie and ES Mentor were obtained in raw and roasted form from the project partner at the Federal Institute of Education and Research Francisco Josephinum (Wieselburg, Austria).

2.2 Chemicals

- 2-Propanol — Honeywell Riedel-de-Haen
- Acetic acid (≥ 99.8 %) for LC-MS — VWR Chemicals BDH
- Acetone (99.5+ %) HPLC grade — Alfa Aesar ThermoFisher
- Acetonitrile (≥ 99.9 %) LC-MS grade — Honeywell Riedel-de-Haen
- AA calibration standard LS100 — Laborservice Onken GmbH
    - 1.25 µmol/mL Cys
    - 2.5 µmol/mL each of Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val, NH_4^+
- Ammonium hydrogencarbonate — Honeywell Fluca
- Apomyoglobin from horse skeletal muscle — SIGMA Life Science
- Bovine serum albumin (≥ 98 %) — Sigma-Aldrich
- Bonsilage PLUS (Granulat) — H. Wilhelm Schaumann GmbH&Co.KG
    - 1k2078 *Lactobacillus plantarum*
    - 1k20711 *Lactobacillus rhamnosus*
    - 1k2103 *Pediococcus pentosaceus*
    - 1k20710 *Lactobacillus brevis*
    - 1k2075 *Lactobacillus buchneri*
- Buffer 1: Sodium Oxidized pH 3.35 — Laborservice Onken GmbH
- Buffer 2: Sodium Hydro/Oxid pH 4.25 — Laborservice Onken GmbH
- Buffer 3: Sodium Oxidized pH 2.65 — Laborservice Onken GmbH
- Buffer 4: Sodium Oxidized pH 8.60 — Laborservice Onken GmbH
- Buffer 6: Sodium Regeneration Buffer — Laborservice Onken GmbH
- Calcium chloride dihydrate — VWR Life Science
- Dimethyl sulfoxide for analysis — MERCK KGaA
- DTT for molecular biology — PanReac AppliChem
- Formic acid (98-100 %) — MERCK KGaA
- Hydrochloric acid (25 %) for analysis — MERCK KGaA
- Hydrochloric acid (37 %) — VWR Chemicals BDH

- Hydrogen peroxide (30 %) for analysis — MERCK KGaA
- Iodoacetamide (≥ 99 %) NMR grade — SIGMA
- L-BAPA (≥ 99 %) — Sigma-Aldrich
- L-(+)-Norleucine (99 %) — Thermo scientific
- Methanol (> 99.9 %) ULTRA LC-MS grade — VWR Chemicals BDH
- n-Hexane (97 %) for HPLC — VWR Chemicals BDH
- Phenol for analysis — MERCK KGaA
- Roti®Quant 5x concentrate — Carl Roth GmbH + Co. KG
- Sodium disulphite (98 %) — VWR Chemicals BDH
- Sodium hydroxide (32 %) in aq. solution — VWR Chemicals BDH
- Thiodiglycol (≥ 95 %) — Sigma-Aldrich
- Trifluoroacetic acid (99.5+ %) HPLC grade — Alfa Aesar
- Tris(hydroxymethyl)-aminomethane — MERCK KGaA
- Trisodium citrate dihydrate — MERCK KGaA
- Trypsin from bovine pancreas — Sigma-Aldrich (for TIA)
- Trypsin Protease (20 µg) MS-Grade — Thermo Scientific (for digestion)
- Urea (99.8 %) — VWR Chemicals BDH

2.3 Solutions

- *Apomyoglobin solution (1 nmol/L)*: First, a vial of 54 nmol apomyoglobin was dissolved in 2 mL ambic (50 mM) and portioned into 5 µL aliquots; one such 5 µL aliquot was then diluted with 125 µL ambic (50 mM).
- *NOR stock solution (10 µmol/mL)*: 328,0 ± 0.1 mg L-(+)-norleucine dissolved in 250 mL buffer solution without NOR.
- *Buffer solution with NOR*: 10 mL NOR stock solution (10 µmol/mL) diluted to 1 L with buffer solution without NOR.
- *Buffer solution without NOR*: 196 g trisodium citrate dihydrate, 200 mL thiodiglycol, 10 g phenol and 150 mL HCl (37 %) combined in 10 L distilled water and adjusted to pH 2.2 ± 0.01 with HCl (25 %).
- *$CaCl_2 \cdot 2H_2O$ solution (0.005 mol/L)*: 735 mg $CaCl_2 \cdot 2H_2O$ dissolved in 1 L HCl (0.001 M).
- *$CaCl_2$/Ambic solution*: $CaCl_2$ (10 mM) diluted 1:10 with ambic (50 mM).
- *DTT solution (0.05 mmol/mL)*: 15.3 mg *DTT* dissolved in 2 mL ambic (50 mM).
- *Elution solution for solid phase extraction*: 60% ACN and 0.2% TFA in ultra-pure water.
- *Extraction solution*: 1 M urea and 0.1 M Tris dissolved in 50 % isopropanol; pH adjusted to 8.2 ± 0.1 with conc. HCl; 0.5 % DTT added right before use.
- *Hydrolysis mixture*: 2 g phenol dissolved in a 1:1 dilution of HCl (37 %) and distilled water.

- *IAA solution (0.1 mmol/mL)*: 18.5 mg IAA dissolved in 1 mL ambic (50 mM); the solution was protected from light by wrapping the container in aluminium foil.
- *L-BAPA solution*: 33-37 mg L-BAPA weighed into a 50 mL volumetric flask, dissolved in 0.5 mL DMSO and filled to the mark with Trisbuffer/$CaCl_2$ solution.
- *Performic acid*: Formic acid (98-100 %) diluted 10:1 with hydrogen peroxide (30 %) and stirred for one hour; for immediate use.
- *Rapigest solution (1 mg/mL)*: A vial of 1 mg Rapigest dissolved in 1 mL ambic (50 mM).
- *Trisbuffer/$CaCl_2$ solution*: 6.05 g Tris and 735 mg $CaCl_2 \cdot 2H_2O$ dissolved in 1 L distilled water; pH adjusted to 8.2 ± 0.1 with HCl (25 %).
- *Trypsin protease solution (0.04 µg/µL)*: A vial of 20 µg trypsin protease dissolved in 40 µL acetic acid (50 mM) and 480 µL $CaCl_2$/ambic solution (for use in chapter Digestion).
- *Trypsin protease solution (0.0135 mg/mL)*: 1 mL trypsin stock solution diluted to 20 mL with $CaCl_2 \cdot 2H_2O$ solution (for use in chapter TIA).
- *Trypsin stock solution (270 mg/L)*: 27 mg trypsin from bovine pancreas dissolved in 100 mL $CaCl_2 \cdot 2H_2O$ solution.
- *Washing solution for solid phase extraction*: 2.5 % ACN and 0.2 % TFA in ultra-pure water.

2.4 Sample preparation

2.4.1 Fermentation

The fermentation set-up was roughly adapted from Lambo et al. (2024)[1]. Raw beans were roughly cracked open using a centrifugal mill by Retsch with a 10 mm sieve grinder for greater surface area during fermentation. Assuming an initial moisture content of around 10 %, 500 g beans were soaked in 200 mL ultra-pure water (40 % v/w) overnight to achieve a moisture content of 50 %. 150 g of the soaked beans were packed into 250 mL-Pyrex bottles, combined with 25 g Bonsilage (10 % w/v), filled to the brim with ultra-pure water and screwed shut to ensure anaerobic conditions. Three biological replicates were prepared per soybean variety. The submerged fermentation took place in an incubator at 37 °C over 72 hours. The fermented beans were dried at 50 °C and grinded to a fine powder once again using a centrifugal mill by Retsch, this time with a 0.5 mm sieve grinder.

2.4.2 Roasting

Raw beans were toasted by the project partner at 225 °C for 290 seconds and grinded to a fine powder with a 0.5 mm sieve grinder.

2.4.3 Proteomics

2.4.3.1 Total extractable protein

Following workflow was adapted from Geisslitz et al. (2022).[18] 100 ± 2 mg of the grinded samples were weighed into 15 mL-falcon tubes in triplicate. Defatting was performed by adding 10 mL n-hexane, vortexing for ten seconds, mixing in an overhead shaker at 40 rpm for five minutes, centrifuging at 4000 rpm for five minutes and discarding the supernatant. This was repeated two more times before the sample was dried with nitrogen gas to evaporate any remaining n-hexane. The proteins were extracted from the degreased samples with 4 mL extraction solution by first displacing the oxygen within the tube with nitrogen to prevent oxidation, then shaking in a water bath at 60 °C for 20 minutes and vortexing halfway in between. The supernatant was collected after centrifugation at 4000 rpm for five minutes and the extraction was repeated once more. Finally, around 2 mL of the combined supernatants were filtered through a regenerated cellulose filter (0.45 µm). 100 µl of the filtered protein extract was mixed with 400 µL cold acetone to precipitate overnight at -20 °C. The acetone was discarded after centrifugation at -4 °C for ten minutes and further cooling at -20 °C for 15 minutes. The remaining protein pellet was washed by adding 400 µl cold acetone, cooled in the freezer at -20°C for one hour and centrifuged as described above. The washing step was repeated, the acetone was discarded, and the pellet was dried with nitrogen gas.

2.4.3.2 Trypsin Digestion

The trypsin digestion workflow was adapted from Geisslitz et al. (2022).[18] The protein pellets were suspended in 50 µL ultra-pure water and mixed with 10 µL Apomyoglobin solution (1 nmol/L) as internal standard, 50 µL Rapigest solution (1 mg/mL) as denaturing agent and 20 µL DTT solution (0.05 mmol/mL) as reducing agent while vortexing between each addition. After incubation in a thermocycler at 60 °C and 300 rpm for 20 minutes, the samples were cooled to room temperature and 20 µL IAA solution (0.1 mmol/mL) was added as an alkylation agent. The samples were incubated in the dark for 30 minutes at room temperature before adding 10 µL DTT solution (0.05 mmol/mL) to inactivate the excess IAA by incubating for another 10 minutes in the dark at room temperature. Finally, 40 µL trypsin protease solution (0.04 µg/µL) was added to the sample solution before incubating in the thermocycler at 37 °C and 300 rpm for 17 hours and 20 minutes. After incubation the digestion was stopped by adding 10 µL TFA (5 %, pH <4).

2.4.3.3 Solid phase extraction

Following workflow was adapted from Geisslitz et al. (2022).[18] The digested samples were purified by solid phase extraction with self-made stage tips using six layers of SDB-XC reversed phase Empore® extraction disks in a 200 µL pipette tip put on a 2 mL Eppendorf tube

with a hole drilled through the cap. The stage tips were loaded with 150 µL methanol and centrifuged at 7000 rpm for three minutes, then loaded with 150 µL washing solution and centrifuged the same way. After the washing step was repeated once more and the Eppendorf tube was emptied, the stage tips were loaded with 200 µL digested extract and centrifuged at 4000 rpm for five minutes. Two washing steps were performed with 200 µL washing solution by centrifuging at 7000 rpm for three minutes. The stage tips were then put on a 1.5 mL LoBind-Eppendorf tube with a hole drilled through the cap, loaded with 200 µL elution solution and centrifuged at 4000 rpm for ten minutes. Finally, the eluates were dried by vacuum centrifugation at 45 °C and 7 torr (10 mbar) for two hours. For the LC-MS measurement, the analytes were dissolved in 100 µL of 10 % ACN and 0.1 % TFA in ultra-pure water.

2.4.4 Trypsin Inhibitor Activity

The trypsin inhibitor activity (TIA) was determined according to the methods specified in OENORM EN ISO 14902:2001[19]. 1,0000-1,0049 g of the grinded samples were weighed into plastic cups in duplicate, suspended in 50 mL NaOH (0,01 M) and stirred on a magnetic stirrer at 320 U/min for 30 minutes. 50 mL distilled water was added to the suspensions before adjusting the pH to 9.5 ± 0.1 with either NaOH (1 M), NaOH (32 %), HCl (1 M) or HCl (25 %). The suspensions were stirred at 320 U/min for two hours before placing in the refrigerator at 4 °C overnight. As a preliminary test, 1:10 dilutions of the sample solutions were prepared to get an estimate for the necessary dilution factor which leads to a 40-60 % inhibition of the sample solution compared to the trypsin standard. The measuring solutions were prepared according to the pipetting scheme in Table 1, then shaken overhead at least 20 times and centrifuged at 14000 rpm for three minutes. The measurements were repeated with adjusted dilutions until the remaining inhibition was in the 40-60 % range.

Table 1: Pipetting scheme for the TIA measuring solutions.

	Blank	**Trypsin std**	**Sample blank**	**Sample**
distilled H_2O	600 µL	600 µL	400 µL	400 µL
diluted sample	-	-	200 µL	200 µL
acetic acid (5.3 M)	200 µL	-	200 µL	-
L-BAPA solution	1 mL	1 mL	1 mL	1 mL
equilibrate for 10 minutes at 37 °C in water bath				
Trypsin solution	200 µL	200 µL	200 µL	200 µL
equilibrate for 12 minutes at 37 °C in water bath				
acetic acid (5.3 M)	-	200 µL	-	200 µL

2.4.5 Soluble protein content

The concentration of soluble proteins in the protein extract was determined by following the Bradford assay[20] described in the Roti®Quant manual by Carl Roth GmbH + Co. KG. First, calibration solutions with the concentrations 0.001 mg/mL, 0.002 mg/mL, 0.005 mg/mL, 0.010 mg/mL, 0.020 mg/mL, 0.050 mg/mL, 0.100 mg/mL and 0.200 mg/mL were prepared using a BSA stock solution (1 mg/mL) and vortexed thoroughly. The filtered protein extract of each sample was diluted 1:10 with extraction solvent – albeit without the DTT – whilst the colour reagent Roti®Quant was diluted 1:5.5 with distilled water and stored in the dark until use. 50 µL of the diluted samples, of the extraction solvent and of distilled water were pipetted into a 96-well plate in triplicate, mixed with 200 µL colour reagent each, then incubated in the dark for five minutes before measurement.

2.4.6 Crude protein content

The crude protein content was determined according to the methods specified in ÖNORM EN ISO 16634-2:2016[21] by combustion of 180-220 mg of the grinded samples tightly packed in aluminium foil and measuring the thermal conductivity of the resulting N_2-gas.

2.4.7 Moisture content

The moisture content was determined in the Moisture Tester MT-CA by Brabender® Measurement & Control Systems according to the instructions of the manufacturer by weighing 10 g of the grinded samples before and after drying at 130 °C for one hour.

2.4.8 Amino acid content

The amino acid composition of the samples was determined according to the methods set out in Annex III.F of the Commission Regulation (EC) No 152/2009[22] with some adjustments. Due to the susceptibility of some amino acids to oxidation, two separate approaches had to be performed simultaneously:

> (n) = “normal“ (unoxidized) approach for the amino acids lysine, threonine, alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, phenylalanine, proline, serine, tyrosine and valine
>
> (ox) = “oxidised” approach for the amino acids cysteine (→ cysteic acid) and methionine (→ methionine sulphone)

For each approach (n and ox), 500-550 mg of the grinded samples were weighed into 250 mL-Pyrex bottles in duplicate. The (ox) samples were suspended in 10 mL freshly prepared performic acid and stirred for one hour on a magnetic stirrer before adding 1.7 g sodium

disulphite. From this point onward, both (ox) and (n) samples were subject to the same treatment. The samples were suspended in 25 mL hydrolysis mixture, stirred well on a magnetic stirrer and shut tightly before placing in an oven at 115 °C for 24 hours. After cooling down, the bottles were opened and filled approximately to the 100 mL mark with buffer solution without NOR. 13 mL NaOH (32 %) were added in a stepwise manner (5+5+3 mL) while stirring for 30 minutes between each addition. The pH was adjusted to 2.2 ± 0.1 with NaOH (32 %) or HCl (25 %), the sample was then stirred for 15 minutes before checking the pH and readjusting to pH 2.2 ± 0.1 if necessary. This process was repeated two or three times until the pH was steady. 2 mL NOR stock solution were pipetted into 200 mL-volumetric flasks before the sample solutions were transferred quantitatively. The flasks were filled to the mark with buffer solution without NOR and shaken well. Around 10-12 mL of the sample solutions were filtered into 15 mL-falcon tubes using regenerated cellulose membrane filters (0.2 µm) after discarding the first few drops. Finally, the (n) samples were diluted 1:10 with buffer solution with NOR whilst the (ox) samples remained undiluted for measurement.

2.5 Analysis

2.5.1 Trypsin Inhibitor Activity

The measurement was performed on the UV-Vis spectrophotometer V-630 by JASCO at 410 nm after an autozero was performed with distilled water. Results were calculated using Formula 1[19] and Formula 2[19].

Formula 1: $$I[\%] = \frac{(A_{Try} - A_B) - (A_S - A_{SB})}{(A_{Try} - A_B)} \cdot 100$$

I Inhibition [%]

A_{Try} Absorption trypsin standard solution

A_B Absorption blank

A_S Absorption sample solution

A_{SB} Absorption sample blank

Formula 2: $$TIA\ \left[\frac{mg}{g}\right] = \frac{I}{100} \cdot \frac{a_{Try}}{a_S} \cdot \frac{p_{Try}}{100}$$

a_{Try} amount of Trypsin in the measuring solution = 0.0027 mg

a_S amount of sample in the measuring solution [g]

p_{Try} purity of trypsin = 56 %

2.5.2 Soluble protein content

The measurement was performed on the PHOmo Microplate Reader by Autobio Diagnostics Co. at 595 nm. Results were calculated by external calibration.

2.5.3 Crude protein content

The measurement was performed on the DuMaster D-480 by BÜCHI, obtaining the nitrogen content from which the protein content can be calculated by using Formula 3[23].

Formula 3: $P[\%] = N[\%] \cdot f$

P Protein content [%]

N Nitrogen content [%]

f Nitrogen-to-protein conversion factor = 6.25

2.5.4 Amino acid content

The measurement was performed on an Amino Acid Analyzer by Biochrom 30Plus based on ion exchange chromatography, subsequent post column derivatization with ninhydrin and VIS-detection at 440 and 570 nm. The gradient of buffer 1 (Sodium Oxidized pH 3.35), buffer 2 (Sodium Hydro/Oxid pH 4.25), buffer 3 (Sodium Oxidized pH 2.65), buffer 4 (Sodium Oxidized pH 8.60) and Buffer 6 (Sodium Regeneration Buffer) for the IC method can be seen in Figure 1. Results were obtained by integration of the peak areas and normalisation to the internal standard norleucine. Quantification was performed using the standard solution LS100.

Sample List | Program

No.	Time	Temp	Buffer	Pump	Nin	Rec	Commands
01	01:00	56°C	3	25.0ml/h	ON	OFF	
02	00:00	56°C	3	25.0ml/h	ON	OFF	Reset
03	01:00	56°C	3	25.0ml/h	ON	ON	Load
04	02:00	56°C	3	25.0ml/h	ON	ON	
05	00:00	56°C	3	25.0ml/h	ON	ON	Reset
06	19:00	56°C	1	25.0ml/h	ON	ON	
07	21:30	58°C	2	25.0ml/h	ON	ON	
08	04:00	58°C	4	25.0ml/h	ON	ON	
09	17:00	80°C	4	25.0ml/h	ON	ON	
10	27:00	90°C	4	25.0ml/h	ON	ON	
11	06:00	90°C	6	25.0ml/h	ON	ON	
12	04:00	90°C	3	25.0ml/h	OFF	ON	
13	02:00	90°C	0	OFF	OFF	OFF	
14	17:00	53°C	3	25.0ml/h	OFF	OFF	
15	03:00	56°C	3	25.0ml/h	ON	OFF	
16	03:00	56°C	3	25.0ml/h	ON	OFF	
End							

Figure 1: IC-Gradient with buffer 1 (Sodium Oxidized pH 3.35), buffer 2 (Sodium Hydro/Oxid pH 4.25), buffer 3 (Sodium Oxidized pH 2.65), buffer 4 (Sodium Oxidized pH 8.60) and Buffer 6 (Sodium Regeneration Buffer).

2.5.5 LC-QTOF-MS

The measurement was performed on an ultra-performance liquid chromatograph (UPLC) coupled with a Quadruple-Time-of-flight high-resolution mass spectrometer (QTOF-HRMS), both from Waters™. The ACQUITYBio H-Class UPLC-System was equipped with a CSH C18 reversed phase column (1 mm x 150 mm, 1.7 µm). The chromatographic separation had a run time of 70 minutes with a flow rate of 0.1 mL/min and column temperature of 60 °C. The gradient of eluent A (0.1 % FA in H_2O) and eluent B (0.1 % FA in 98 % ACN and 2 % H_2O) is shown in Figure 2, the complete LC method in Figure 3. The Xevo G2 XS QTOF HRMS-system equipped with electrospray ionisation (ESI) was used for the detection of the analytes .

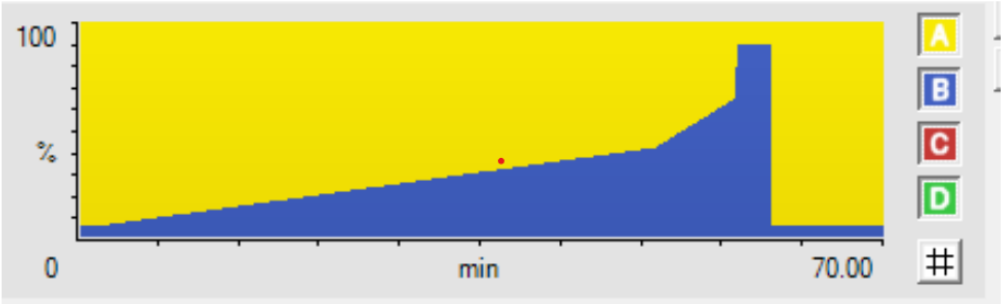

Figure 2: LC-gradient with eluents A (0.1 % FA in H_2O) and B (0.1 % FA in 98 % ACN and 2 % H_2O).

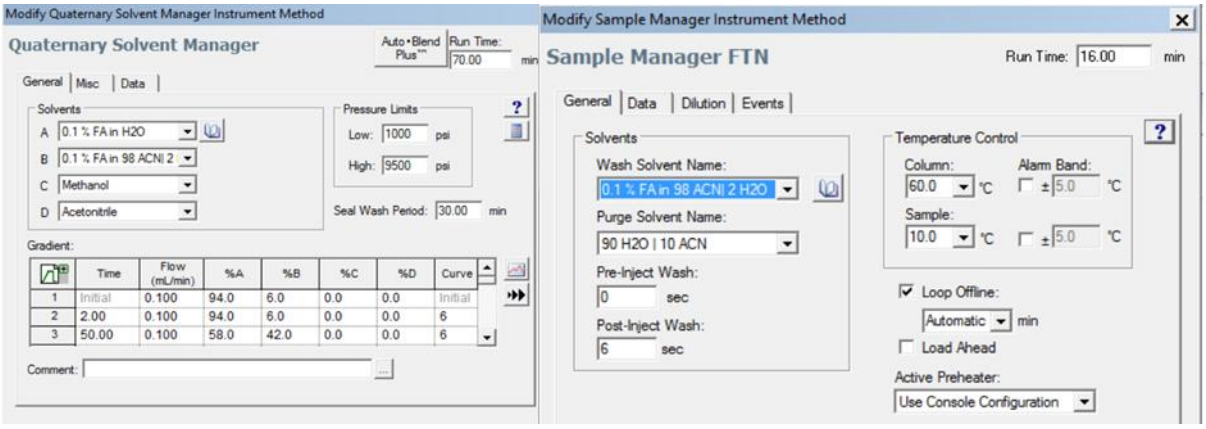

Figure 3: Complete LC-method.

For peptide identification and relative quantification, Data Independent Acquisition (DIA) in SONAR mode from 400-1200 m/z with an isolation mass window of 35 Da was applied. The measurement was performed in positive ionisation. The settings for the ESI source of the QTOF, which were applied for DIA measurements, are listed in Table 2.

Table 2: Settings for DIA measurement with Xevo G2 XS QTOF.

QTOF	Settings
Capillary	2.8 kV
Sampling cone	30 V
Source offset	60 V
Source	150 °C
Desolvation	450 °C
Cone gas flow	50 L/h
Desolvation gas flow	500 L/h

The DIA data was processed with the software tool ProteinLynx Global Server (PLGS) from Waters. All acquired data was first processed with Apex 3D within PLGS where it was converted from continuum to monoisotopic data. Table 3 shows the parameters used for processing.

Table 3: Processing parameters for Apex 3D.

Attribute	Value
Chromatographic peak width	Automatic
MS TOF Resolution	Automatic
Lock Mass for Charge 1	556.2771 Da/e
Lock Mass Window	0.25 Da
Low Energy Threshold	60.0 counts
Elevated Energy Threshold	15.0 counts

Protein identification of the processed DIA data was performed with imported databases from UniProtKB for soybeans. A FastaFile with 777 reviewed entries was used. Table 4 shows the workflow parameters for the databank search query.

Table 4: Workflow parameters for Databank Search Query.

Attribute	Value
Search Engine Type	PLGS
Databank	Soybean FastaFile (777 proteins)
Peptide Tolerance	Automatic
Fragment Tolerance	Automatic
Min Fragment Ion Matches per Peptide	3
Min Fragment Ion Matches per Protein	7
Min Peptide Matches Per Protein	1

Maximum Protein Groups to Return	20
Maximum Protein Mass	250000
Primary Digest Reagent	Trypsin
Missed Cleavages	1
Fixed Modifier Reagents	Carbamidomethyl C
Variable Modifier Reagents	Glycation N-Term, Oxidation M
False Discovery Rate	4
Calibration Protein	P68082
Calibration Protein Concentration	1558
Monoisotopic Or Average	Monoisotopic
Peptide Charge	1+
Instrument Type	ESI-QUAD-TOF

For relative quantification, the peak areas of pre-selected unique peptides listed in Table 5 were normalised to the internal standard apomyoglobin (P68082) and summed up per protein, then normalised to the weight. The resulting protein peak areas were averaged per set of technical replicates, excluding any values identified as outliers via the Dean-Dixon Q test. As three biological replicates were performed of the fermentation, three separate fermentation results were obtained. To obtain an average result for the fermentation experiment, the respective technical replicates were tested for an outlier using the Dean-Dixon Q test and then averaged per biological replicate accordingly. Next, those results were evaluated statistically by one-way analysis of variance (ANOVA) with post-hoc Tukey HSD test ($\alpha = 0.05$) using IBM SPSS Statistics 29 – only the biological replicates that were not outliers and fell into the same statistical group were averaged and presented as mean ± standard deviation (SD) under the label “fermented”. Thus, a relative content comparison between raw, roasted and fermented samples could be performed. Rare cases where the biological replicates provided such highly variable results that they could not be averaged are highlighted separately. The relative content of each analysed protein was compared between the soybean varieties as well.

Table 5: Selected Unique Peptides for the untargeted method.

Protein	UniProt ID	Unique Peptides
Glycinin G1	P04776	VLIVPQNFVVAAR GGLSVIKPPTDEQQQRPQEEEEEEEDEKPQCK VFDGELQEGR ALIQVVNCNGER IESEGGLIETWNPNNKPFQCAGVALSR GIFGMIYPGCPSTFEEPQQPQQR

Glycinin G2	P04405	NNNPFSFLVPPQESQR QQEEENEGSNILSGFAPEFLK VFDGELQEGGVLIVPQNFAVAAK LSAQYGSLR
Glycinin G3	P11828	QQEEENEGGSILSGFAPEFLEHAFVVDR FYLAGNQEQEFLQYQPQK VFDGELQEGQVLIVPQNFAVAAR GGLSVISPPTEEQQQRPEEEEKPDCDEK
Glycinin G4	P02858	VFYLAGNPDIEYPETMQQQQQQK QGQHQQEEEEEGGSVLSGFSK AIPSEVLAHSYNLR QFQLSAQYVVLYK WQEQQDEDEDEDEDDEDEQIPSHPPR GQLLVVPQNFVVAEQAGEQGFEYIVFK YEGNWGPLVNPESQQGSPR VESEGGLIQTWNSQHPELK HFLAQSFNTNEDIAEK GALGVAIPGCPETFEEPQEQSNR
Glycinin G5	P04347	VESEGGLIETWNSQHPELQCAGVTVSK VFYLAGNPDIEHPETMQQQQQQK QFGLSAQYVVLYR FNECQLNNLNALEPDHR YQGNSGPLVNP
β-conglycinin α (subunit 1 and 2)	P0DO15 P0DO16	NFLAGSQDNVISQIPSQVQELAFPGSAQAVEK LITLAIPVNKPGR VPSGTTYYVVNPDNNENLR NPFLFGSNR
β-conglycinin β (subunit 1 and 2)	P25974 F7J077	QVQELAFPGSAQDVER DLDIFLSSVDINEGALLLPHFNSK SSNSFQTLFENQNGR TISSEDEPFNLR QQEGVIVELSK EDENNPFYLR VLFGEEEEQR VLLGEEEEQR
Basic 7S globulin 1	P13917	TPLMQVPVLVDLNGNHLWVNCEQQYSSK GAIIFGDAPNNMR

		CADLFNFANA
Basic 7S globulin 2	Q8RVH5	CGDLFNFANA
β-amylase	P10538	LSMFGVTYLR EYLTVGVDNEPIFHGR AGHPEWELPDDAGK
Kunitz-type trypsin inhibitor KTI1	P25272	EICPLTVVQSPNELDK CEDIGIQIDDDGIR
Trypsin inhibitor A Trypsin inhibitor B KTI3	P01070 P01071	LVFCPQQAEDDK FIAEGHPLSLK GIGTIISSPYR VSDDEFNNYK CGDIGISIDHDDGTR
Bowman-Birk type proteinase inhibitor C-II	P01063	CLDTTDFCYKPCK SDHSSSDDESSKPCCDLCMCTASMPPQCHCADIR
Bowman-Birk type proteinase inhibitor D-II	P01064	CLDTNDFCYKPCK SDQSSSYDDDEYSKPCCDLCMCTR

3 Results and discussion

3.1 Crude protein content

The crude protein content (CPC) was compared between the soybean varieties, as seen in Figure 4. The measured values range around 39-42 % on dry matter basis, which is in line with values from literature[6]. The variety Atacama is at the higher end of the range while the variety Mentor is at the lower end. As results were obtained in single determination, no statistical evaluation could be made.

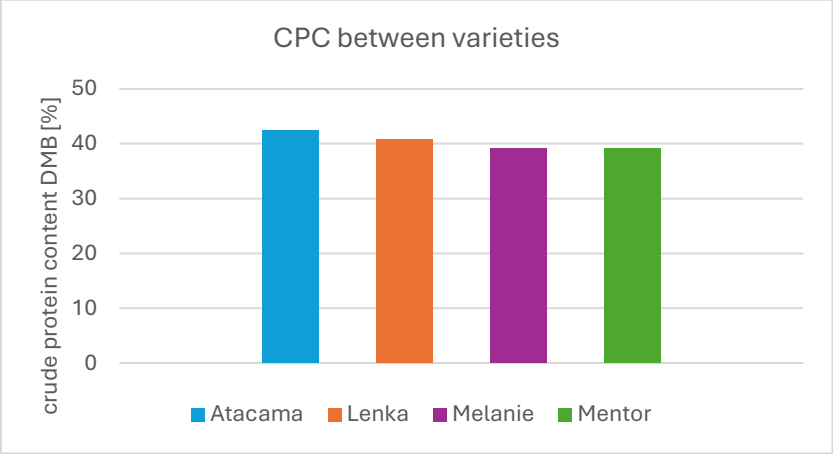

Figure 4: CPC comparison between raw soybean varieties.

The CPC was then compared between raw, roasted and fermented soybeans per variety. No significant difference was observed between the raw and roasted samples while the fermented samples showed a slight reduction across all varieties, with CPC values of 35.4 ± 1.41 % (Atacama), 33.8 ± 1.04 % (Lenka), 32.6 ± 0.32 % (Melanie) and 32.2 ± 0.03 % (Mentor). These results indicate that the CPC was not affected by the roasting process. This was also observed in multiple studies where the crude protein content did not decrease after roasting, particularly at lower temperatures ranging from 110-145 °C.[3,14,24] While the fermented samples showed a lower CPC than the raw samples by approximately 17 %, many studies have reported an increase of the crude protein content [25–27], namely by up to 18 %[27]. The decrease of the CPC observed in the fermented samples of this work may be explained by protein hydrolysation during metabolic activities of the fermenting microorganism.[28]

3.2 Soluble protein content

The soluble protein content (SPC) determined by the Bradford method – and not by the standard practice set out in ISO 14244:2014[29] – was compared between the soybean varieties, as seen in Figure 5. The measured values range around 22-24 % on dry matter basis. No significant difference in the SPC was observed between the varieties Atacama and Mentor

when a One-Way ANOVA with post-hoc Tukey HSD test (α = 0.05) was performed, while the variety Melanie measured slightly higher and the variety Lenka showed an intermediate value.

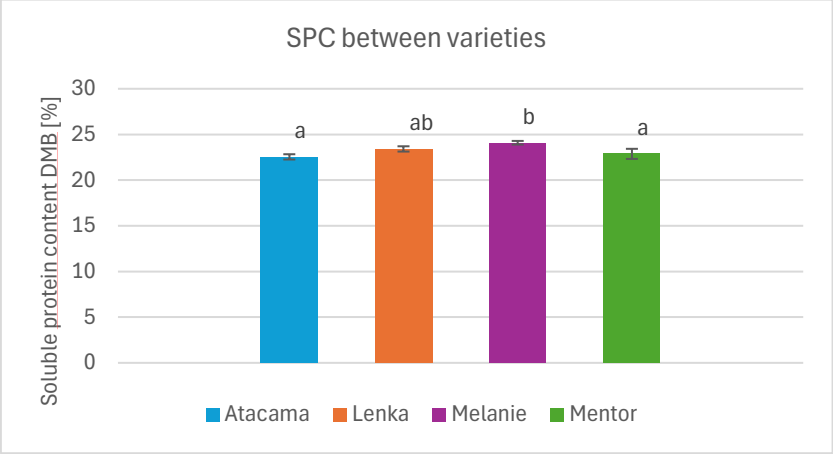

Figure 5: SPC comparison between raw soybean varieties; variables with same letter = no statistically significant difference (α = 0.05).

A study involving over 400 different soybean varieties reported an average SPC of 27 g per 100 g of total protein, with results ranging from 20 to 40 g/100 g across the samples. Variations in SPC between varieties are thought to be correlated to their different glycinin/β-conglycinin ratios.[30] The SPC results above are within the reported range mentioned previously. Subsequently, SPC was compared between raw, roasted and fermented samples per variety. Both roasting and fermentation led to a notable decrease in the SPC, with roasting causing a higher loss than fermentation in three out of four varieties. While the variety Atacama recorded no statistically significant difference between its roasted (17.4 ± 0.4 %) and fermented samples (17.9 ± 0.5 %), the variety Lenka (roasted: 17.8 ± 0.7 %; fermented: 19.3 ± 0.3 %) the variety Melanie (roasted: 17.7 ± 0.2 %; fermented: 20.0 ± 0.5 %) and the variety Mentor (roasted: 18.0 ± 0.4 %; fermented: 20.8 ± 0.6 %) did. The results of the variety Atacama and the variety Lenka can be seen in Figure 6 and Figure 7 respectively.

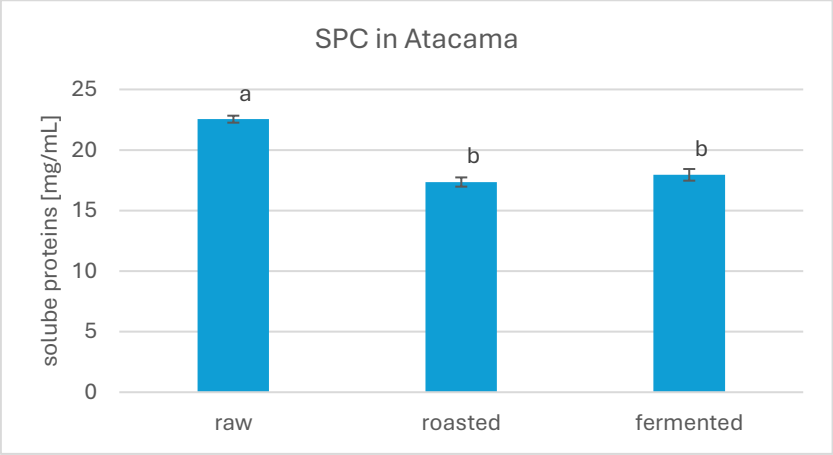

Figure 6: SPC comparison between raw, roasted and fermented Atacama samples; variables with same letter = no statistically significant difference (α = 0.05).

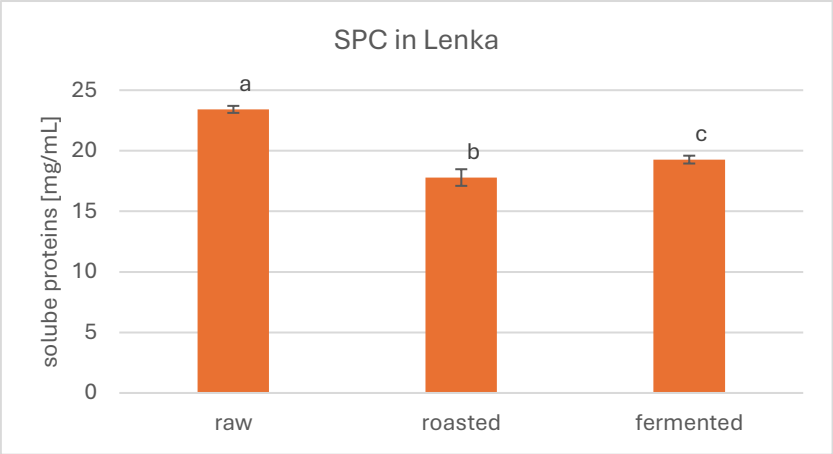

Figure 7: SPC comparison between raw, roasted and fermented Lenka samples; variables with same letter = no statistically significant difference (α = 0.05).

The highest reduction in the SPC was observed in the roasted Melanie sample, exhibiting an approximate decrease of 27 %. Multiple studies have also recorded a decrease of the protein solubility after heat treatments by up to 96 %[27] caused by denaturation and thermal aggregation of glycinin and β-conglycinin to insoluble aggregates[31]. A loss of the SPC was observed in fermentation studies as well, namely up to 35 % in the case of fungal fermentation[27] or even 90 % in soy protein isolates fermented by *Lactobacillus plantarum* strains[32]. This decrease is presumed to have been caused by the acid produced by the bacteria inducing protein denaturation and formation of insoluble protein aggregates.[32] However, it is to note that these fermentation studies include a heated pre-treatment of the soybeans, which was not applied to the samples of this work. This methodological difference may explain the different rates of SPC reduction when comparing the values with literature, as TIs are known to be inactivated during moist heat treatments[9]. There are also studies that have recorded an increase of the SPC by up to 63 % due to strong hydrolysis of the proteins during fermentation.[25] Nonetheless, the results obtained from the roasting and fermentation experiments show that the initial SPC undergo a significant reduction after both treatments.

3.3 Amino acid content

The amino acid content (AAC) was compared between the soybean varieties in their raw form, as seen in Figure 8. A One-Way ANOVA with post-hoc Tukey HSD test (α = 0.05) was performed. No statistically significant differences in the content was observed between the varieties for eleven out of the 17 amino acids. However, serine, isoleucine, leucine, tyrosine, histidine, and arginine, exhibited slightly but statistically lower concentrations in certain varieties. Overall, no substantial difference in the AAC was detected among the varieties examined in this work. Comparable results were reported in a separate study were 55 soybean samples from different regions showed no statistically significant difference between their total amino acid compositions.[33]

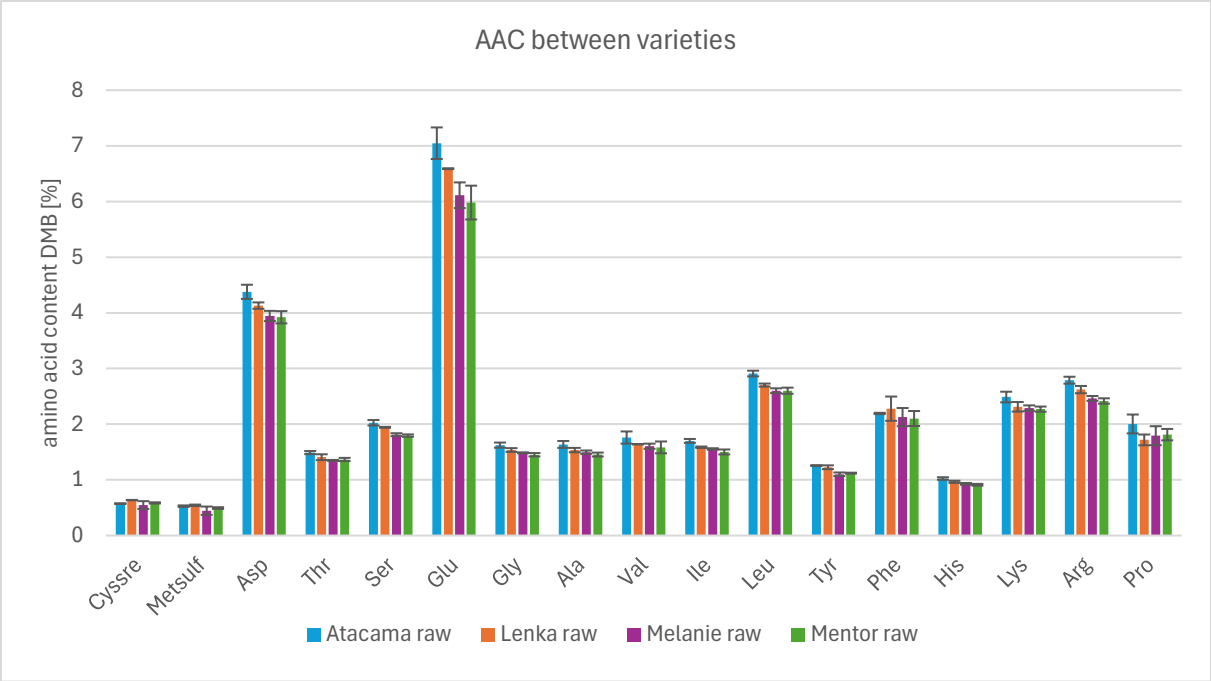

Figure 8: AAC comparison between raw soybean varieties.

An additional statistical analysis was performed to compare the AAC across raw, roasted and fermented soybeans within each variety. The results can be seen in Figure 9 for the variety Atacama, Figure 10 for the variety Lenka, Figure 11 for the variety Melanie and Figure 12 for the variety Mentor. The samples statistically confirmed to contain the lowest amount of a specific amino acid are marked with a red symbol (*). For the majority of amino acids, no difference was observed between raw and roasted samples. In contrast, the fermented samples consistently recorded the lowest amounts across all varieties, with only minor exceptions. For example, alanine shows no statistically significant difference between the raw, roasted and fermented samples of the varieties Atacama, Lenka and Mentor whereas a significant reduction was observed in the fermented Melanie sample. Statistically significant differences between raw and roasted samples were rare and even fewer cases showed no significant difference between roasted and fermented samples. Overall, no drastic change in the amino acid composition was observed after either one of the treatments; only in some cases did the amounts vary by slight yet statistically significant margins.

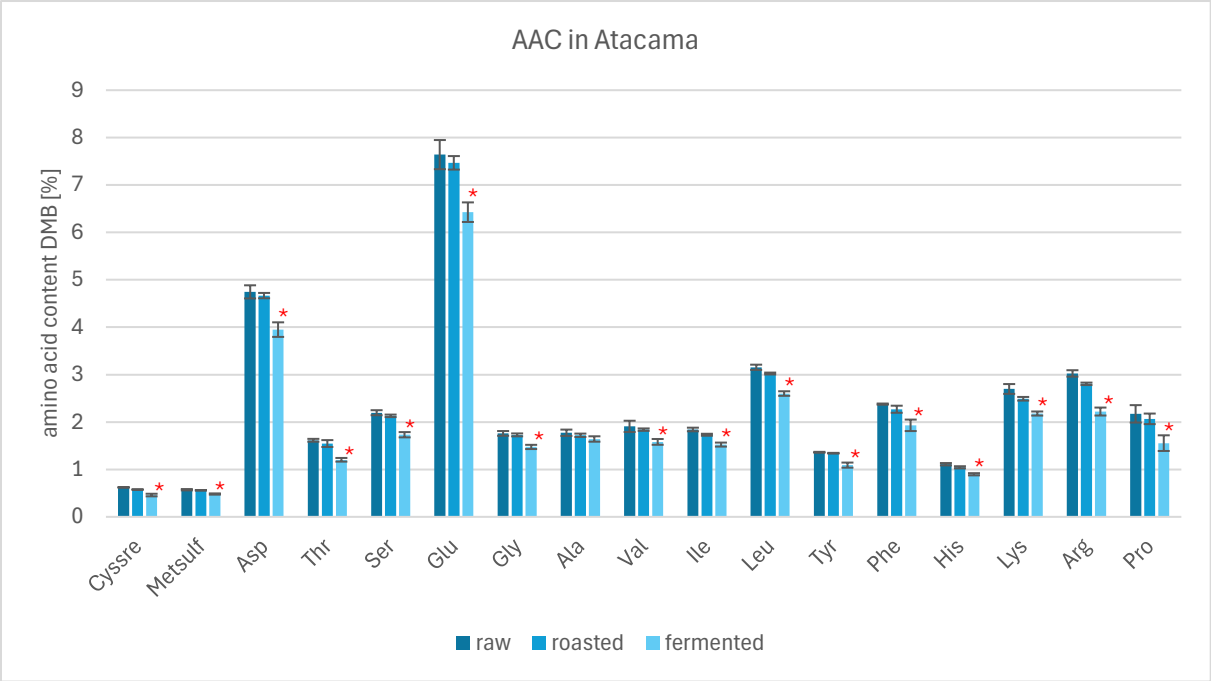

Figure 9: AAC comparison between raw, roasted and fermented Atacama samples; symbol (*) = statistically confirmed lowest content.

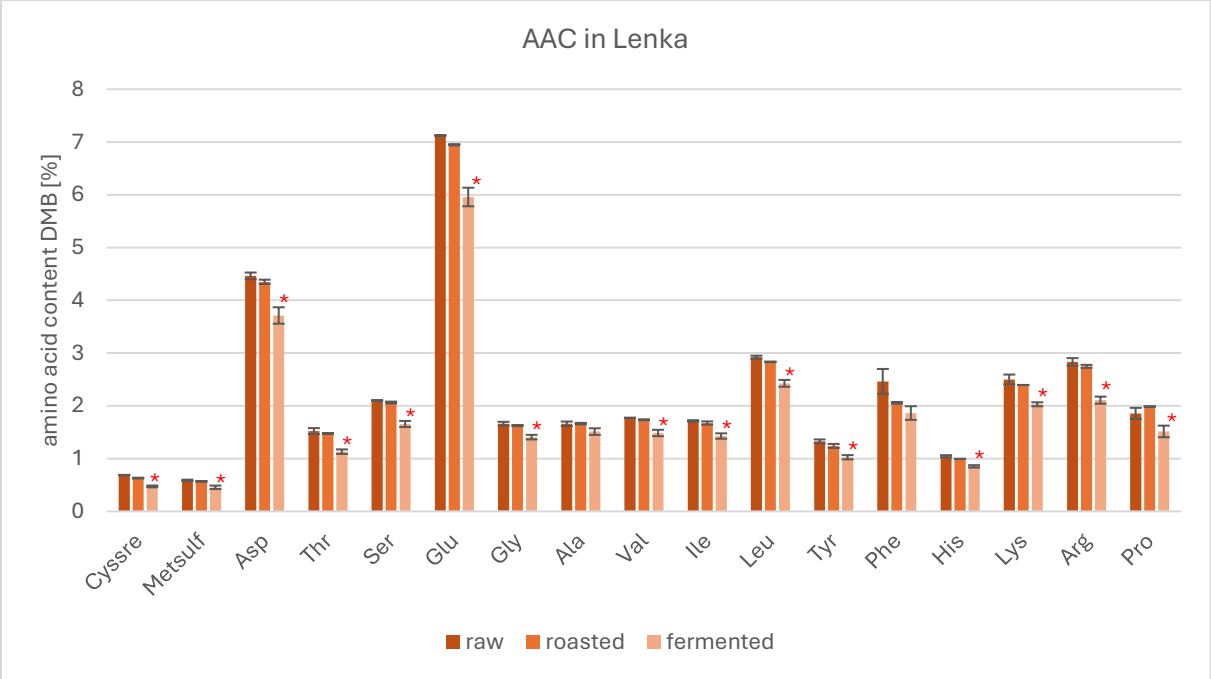

Figure 10: AAC comparison between raw, roasted and fermented Lenka samples; symbol (*) = statistically confirmed lowest content.

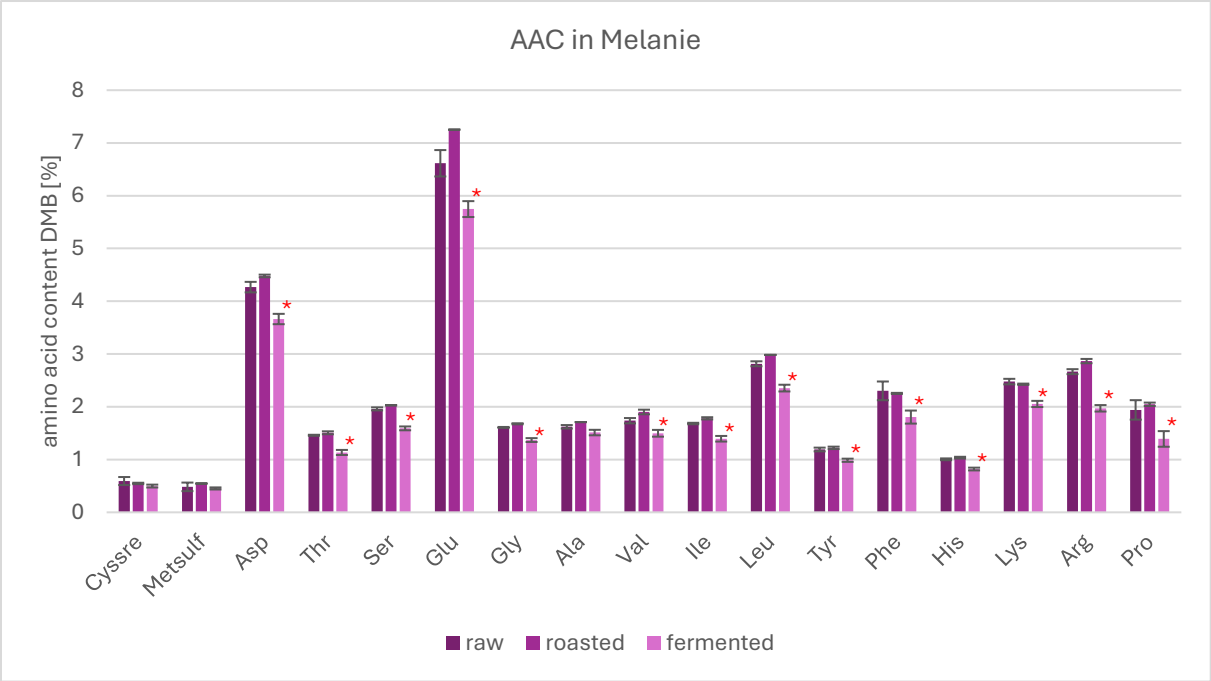

Figure 11: AAC comparison between raw, roasted and fermented Melanie samples; symbol (*) = statistically confirmed lowest content.

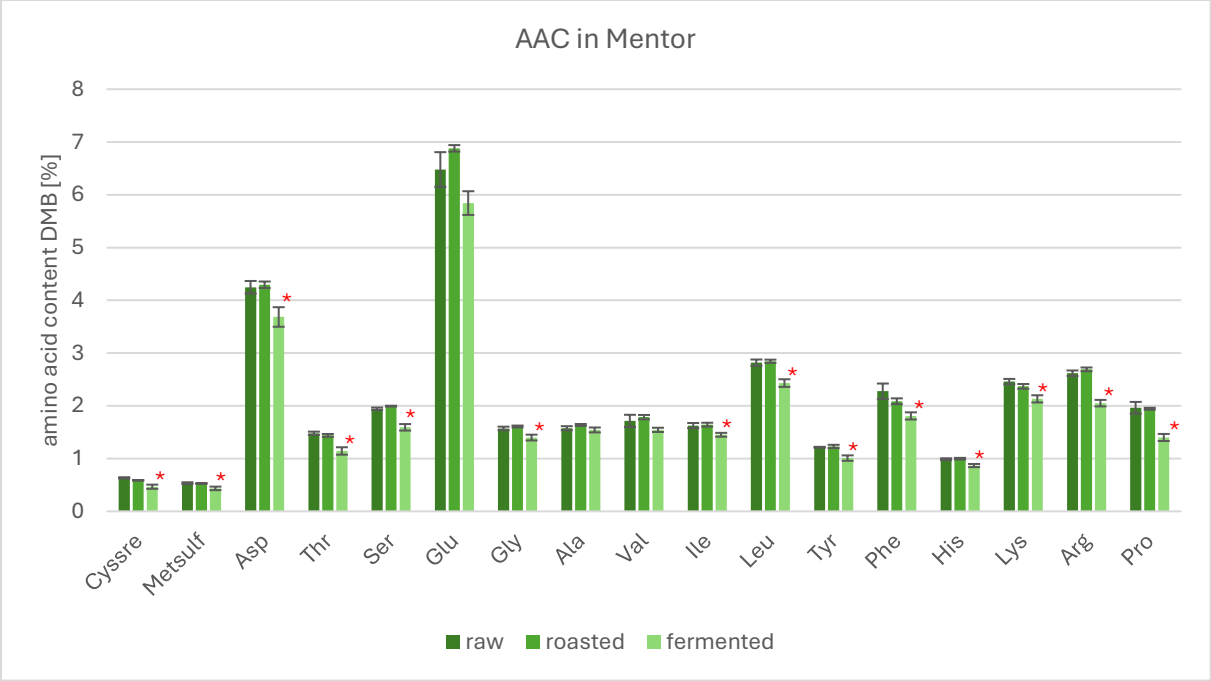

Figure 12: AAC comparison between raw, roasted and fermented Mentor samples; symbol (*) = statistically confirmed lowest content.

Contrary to these results, literature often reports an increase of the AAC. To give an example, one study utilizing *Lactobacillus plantarum* for fermenting soybean meal recorded a statistically significant increase of the amino acids glutamic acid, aspartic acid, alanine, serine, lysine, threonine, tyrosine and phenylanine.[34] In comparison, these previously mentioned amino acids bar a few exceptions were significantly reduced in the fermented samples of all four varieties investigated within this work; only glutamic acid and phenylalanine were reduced in three

varieties each while alanine was reduced in just one variety. However, it is to note that the fermentation method applied in this work differs from the one mentioned in the study, which may have caused the discrepancy. The observed decrease may be caused by the degradation and utilization of the amino acids by the fermenting microorganism.[35] The findings of this chapter correspond to the results from chapter 3.1, as no significant difference in the CPC was observed between the raw and roasted samples of any variety, meanwhile a notable decrease of the CPC was recorded in the fermented samples.

3.4 Trypsin Inhibitor Activity

The trypsin inhibitor activity (TIA) was compared between the soybean varieties in their raw form. As seen in Table 6, the measured values range between 20-29 mg/g, which is in line with values reported in literature.[36] No statistically significant difference in the TIA was observed between the analysed varieties when a One-Way ANOVA with post-hoc Tukey HSD test ($\alpha = 0.05$) was performed.

Table 6: TIA comparison between raw soybean varieties.

Variety	TIA [mg/mL]
Atacama	20.9 ± 0.7
Lenka	28.8 ± 1.8
Melanie	26.2 ± 1.4
Mentor	26.8 ± 1.5

Next, the TIA of the raw samples was compared to the TIA of the roasted and fermented samples per variety. A significant drop of the TIA is observed in the roasted samples, with all four varieties falling below the suggested upper threshold for animal feed of 4 mg/g[13]. The fermented samples did not achieve such low levels, only reaching TIA levels of 8.1 ± 0.8 mg/g (Atacama), 12.0 ± 1.4 mg/g (Lenka), 13.6 ± 0.2 mg/g (Melanie) and 11.2 ± 1.1 mg/g (Mentor). The very similar pattern resulting for each variety can be exemplified by the results of the variety Atacama, visualised in Figure 13.

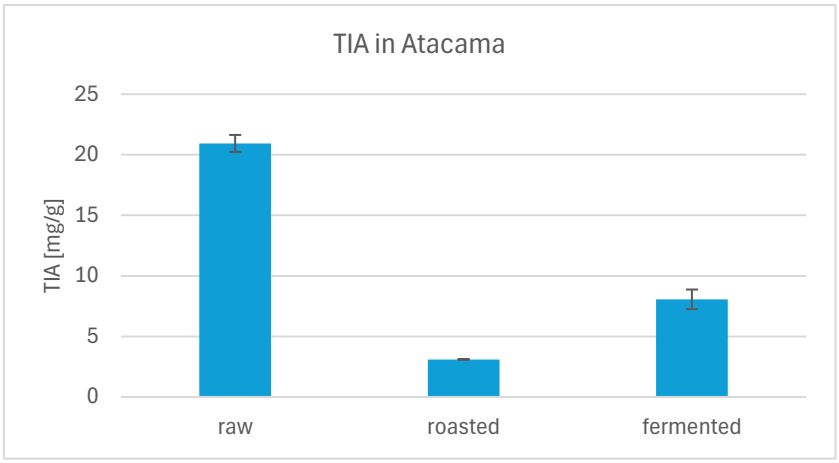

Figure 13: TIA comparison between raw, roasted and fermented Atacama samples.

The most pronounced reduction in the TIA was observed in the roasted sample of the variety Lenka, with a decrease of 92 % compared to the raw sample. The greatest TIA loss among the fermented samples was by roughly 62 % for the variety Atacama. Many studies have reported a decrease in the TIA by up to 93 % after roasting[3] and 96 % after fermentation[25] while an increase of the protein content was recorded at the same time.[3,25,26] However, these fermentation studies include an autoclaving step at up to 120 °C before the samples are fermented[25,26], which may have affected the TIA levels as TIs can get inactivated during moist heat treatments[9]. Nonetheless, this puts forward that TIs are rather inactivated than completely destroyed, namely by structural changes affecting their functional properties[37], as no loss of the protein content is observed. The pre-treatment utilising heat could explain the difference between the TIA reported for fermented samples in literature and the recorded values within this work.

3.5 Proteomics

3.5.1 Comparison of the relative protein content between soybean varieties

By employing an untargeted LC-MS approach, the relative content of selected soybean proteins (Table 5) was investigated between varieties. For this, the peak area of a protein was compared between the soybean varieties in their raw form, as seen in Figure 14 for glycinin G1. No statistically significant difference was observed between varieties when a One-Way ANOVA with post-hoc Tukey HSD test ($\alpha = 0.05$) was performed.

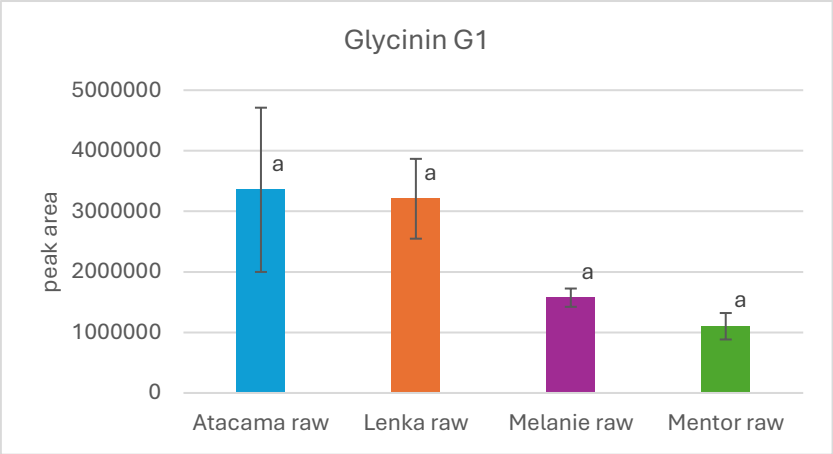

Figure 14: Relative comparison of the glycinin G1 content between raw soybean varieties; variables with same letter = no statistically significant difference (α = 0.05).

The glycinin subunits G2 and G5 exhibited a similar pattern to glycinin G1, with no statistically significant difference among the soybean varieties. In contrast, subunits G3 and G4 showed notable variation. As shown in Figure 15, the variety Lenka has a higher glycinin G3 content compared to the varieties Atacama and Mentor, which belong to the same statistical group. The variety Melanie displayed intermediate levels, showing no statistically clear distinction from any other variety.

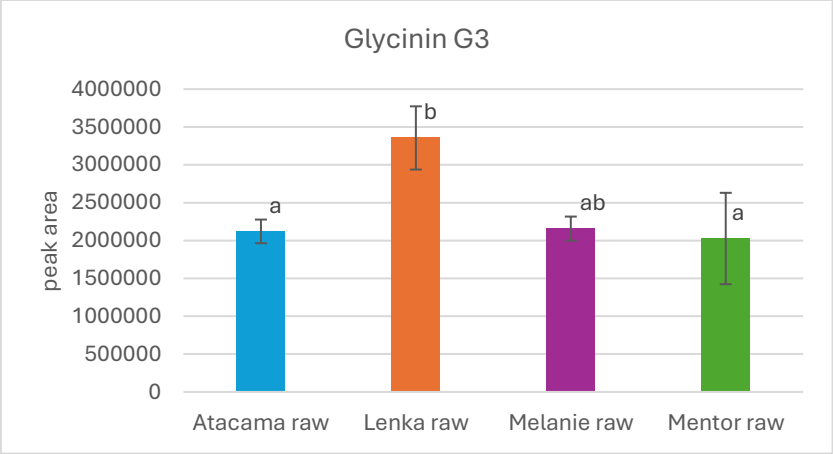

Figure 15: Relative comparison of the glycinin G3 content between raw soybean varieties; variables with same letter = no statistically significant difference (α = 0.05).

As shown in Figure 16, the glycinin G4 displays the highest concentration in the variety Atacama and the lowest in Mentor, while the varieties Lenka and Melanie displayed intermediate values. These results are consistent with the findings observed in Chapter 3.1 Figure 4, where the CPC of the variety Atacama was at the higher end of the recorded range and the variety Mentor at the lower end, closely followed by the variety Melanie.

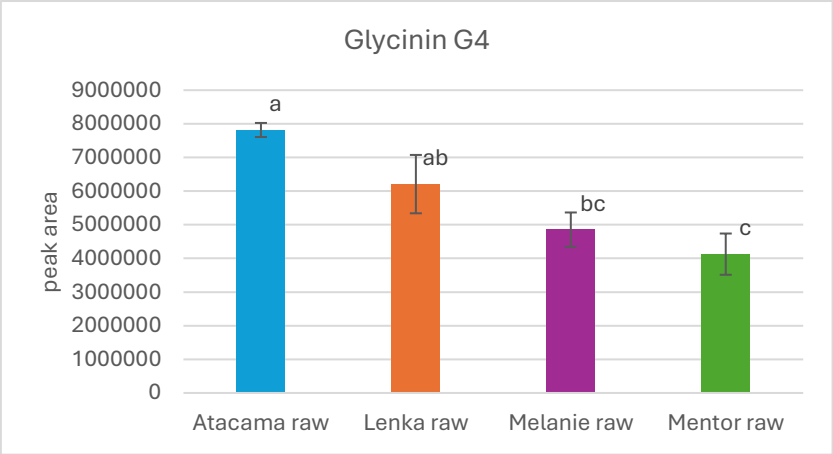

Figure 16: Relative comparison of the glycinin G4 content between raw soybean varieties; variables with same letter = no statistically significant difference (α = 0.05).

The content of the β-conglycinin α protein, seen in Figure 17, appears to be lowest in the variety Mentor, although the variety Atacama falls within the same statistical range. The varieties Atacama, Lenka, and Melanie are grouped together statistically, indicating comparable levels of this particular protein. No significant difference was observed among any of the varieties in the β-conglycinin β content.

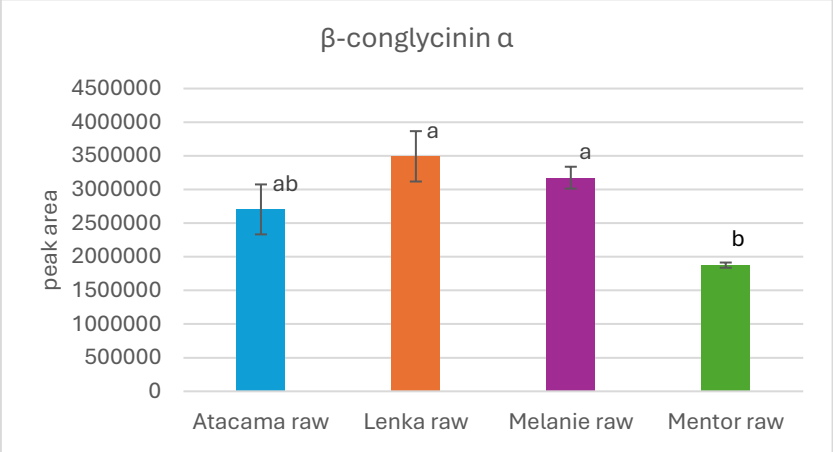

Figure 17: Relative comparison of the β-conglycinin α content between raw soybean varieties; variables with same letter = no statistically significant difference (α = 0.05).

The variety Mentor recorded the lowest values in two of the previously discussed parameters, which may explain its lower CPC as glycinins and β-conglycinins represent the most abundant protein fractions in soy[5]. Additionally, the variety Mentor also recorded the lowest SPC. However, the glycinin content is reported to be negatively correlated with protein solubility, as its rigid structure limits its soloubility.[30] This correlation, however, could not be observed in the present work.

The content of both basic 7S globulin 1 and basic 7S globulin 2 do not differ statistically among the varieties, as exemplified by the results for basic 7S globulin 1in Figure 18. Similarly, no statistically significant differences were observed for β-amylase as well as both BBI proteins investigated within this work, namely the Bowman-Birk type proteinase inhibitor C-II and D-II.

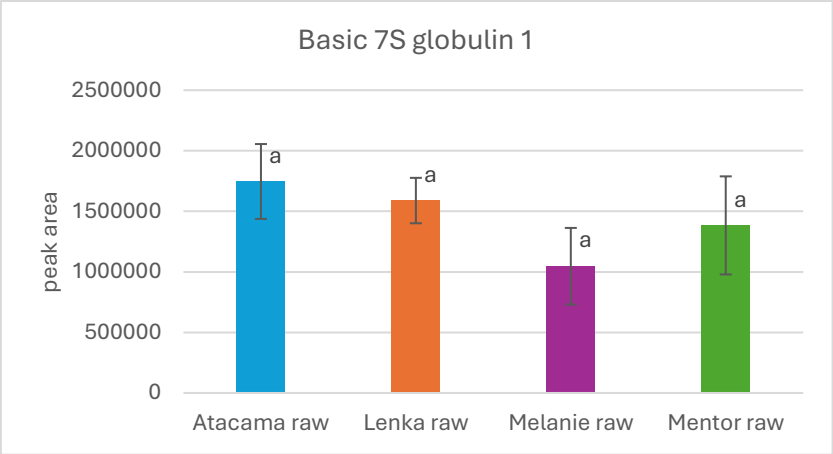

Figure 18: Relative comparison of the basic 7S globulin 1 content between raw soybean varieties; variables with same letter = no statistically significant difference (α = 0.05).

The Kunitz-type trypsin inhibitor KTI1 displays a similar pattern to that of glycinin G4 protein, with the variety Atacama having the highest amount and Mentor the lowest, seen in Figure 19, while the varieties Lenka and Melanie display intermediate values.

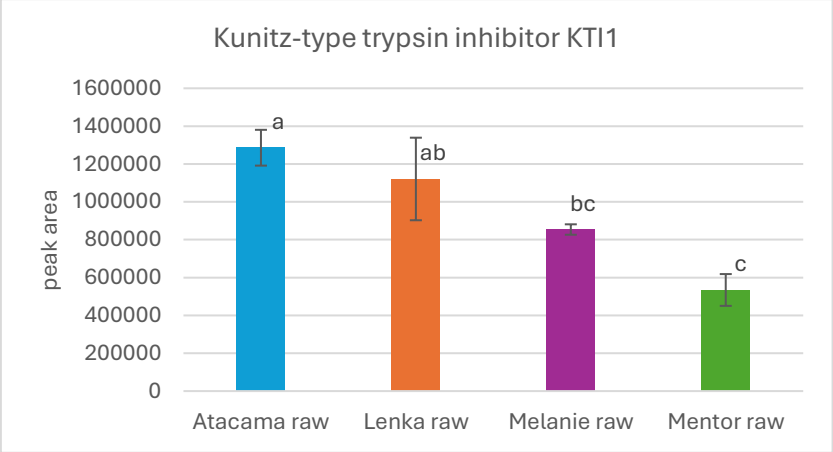

Figure 19: Relative comparison of the Kunitz-type trypsin inhibitor KTI1 content between raw soybean varieties; variables with same letter = no statistically significant difference (α = 0.05).

The Kunitz-type trypsin inhibitor KTI3 is present in statistically similar amounts across all four soybean varieties. Consequently, of the 14 proteins analysed, only four – glycinins G3 and G4, β-conglycinin α, and KTI1 – exhibited statistically significant variation among the varieties. The remaining proteins showed no statistically significant differences in their amounts, indicating very similar protein profiles of the soybean varieties. The variety Mentor recorded the least amount of a protein in three instances, two of which are major seed storage proteins, resulting in the lowest CPC among the four varieties.

3.5.2 Comparison of the relative protein content between raw, roasted and fermented soybean samples

By employing an untargeted LC-MS approach, the change in the content of selected soybean proteins (Table 5) was investigated after roasting and fermentation per variety. As seen in

Figure 20, no statistically significant difference of the glycinin G1 content was observed between the samples of the variety Atacama when a One-Way ANOVA with post-hoc Tukey HSD test (α = 0.05) was performed. The same result was found for the varieties Lenka and Mentor.

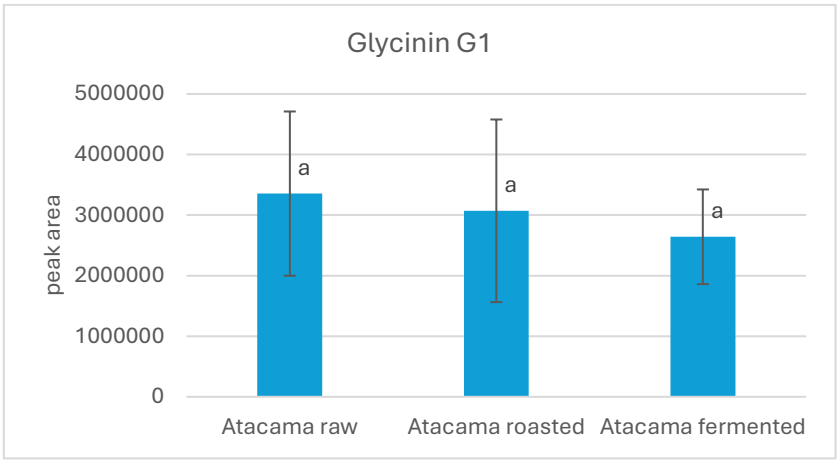

Figure 20: Relative comparison of the glycinin G1 content between raw, roasted and fermented Atacama samples; variables with same letter = no statistically significant difference (α = 0.05).

However, a notable result was obtained for the variety Melanie, as seen in Figure 21. The height pattern of the bars suggests that the roasted sample has more glycinin G1 than the initial amount in the raw sample. Furthermore, the biological replicates of the fermentation experiment provided such different results from each other that no average could be calculated. These results highlight how variable the reproducibility of a complex biological process such as fermentation can be.[4] The results of the variety Melanie were therefore excluded from the evaluation of glycinin G1.

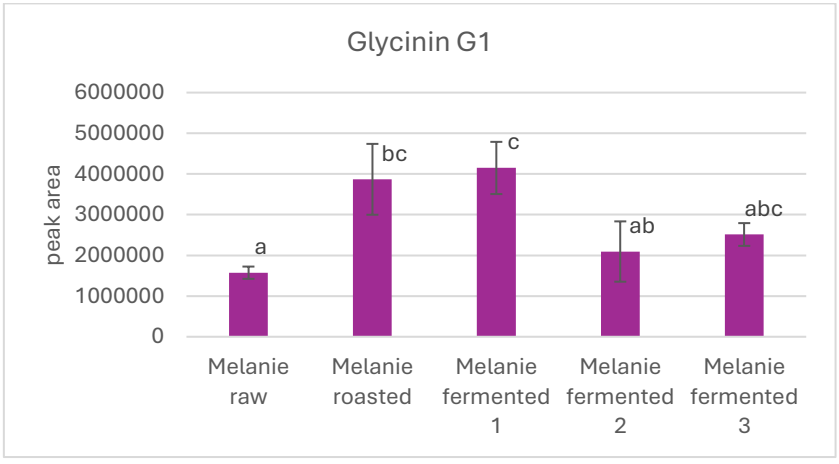

Figure 21: Relative comparison of the glycinin G1 content between raw, roasted and fermented Melanie samples; variables with same letter = no statistically significant difference (α = 0.05).

Considering only the varieties Atacama, Melanie and Mentor, the results suggest that glycinin G1 is a relatively stable protein not affected by either roasting or fermentation, at least not in a statistically significant way. Glycinin is known for its rigid structure, stabilised by disulfide bonds[30], which may explain the stability. In the case of glycinin G2, a similar pattern to that

shown in Figure 9 is displayed in all varieties except one. While the relative glycinin G2 content in the samples of the varieties Lenka, Melanie and Mentor did not change in a statistically significant way after either treatment, a pronounced difference was detected in the variety Atacama, as seen in Figure 22.

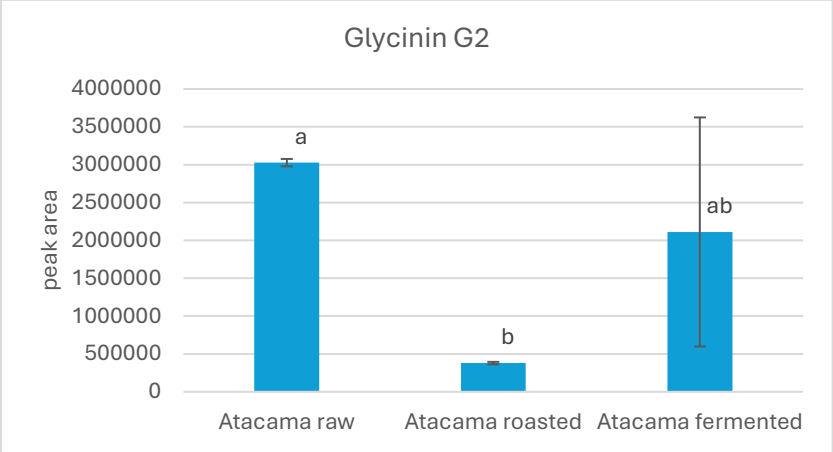

Figure 22: Relative comparison of the glycinin G2 content between raw, roasted and fermented Atacama samples; variables with same letter = no statistically significant difference (α = 0.05).

The notably low peak area of the roasted sample in Figure 22 seems to be an anomaly, as no comparable behaviour was observed in the other varieties. Additionally, the large SD in the glycinin G2 content of the fermented sample indicates a substantial variability in the data, resulting in an overlap of statistical groups which would perhaps be distinct otherwise, due to Tukey's HSD being a conservative test requiring a strong difference to declare significance[38]. Since no significant changes were observed in the varieties following treatments, glycinin G2 seems to be a stable protein similar to glycinin G1. This was an expected result as glycinin subunits are categorized into two groups based on their sequence homology – namely group I (G1, G2, G3) and group II (G4, G5), sharing a sequence identity of 80 % each – which could explain their similar (thermal) stabilities.[39] A case where the large SD changes the statistical grouping of the samples is seen in Figure 23, displaying the results of the glycinin G3 protein in the variety Mentor. The statistical group labels in Figure 23 suggest no significant differences among the Mentor samples, likely due to the large SD caused by highly variable data of the raw sample overlapping with the range of the others. However, a similar height pattern of the bars is observed in Figure 24 displaying the glycinin G3 results of the variety Atacama, where there is indeed a statistically significant difference between the raw and treated samples.

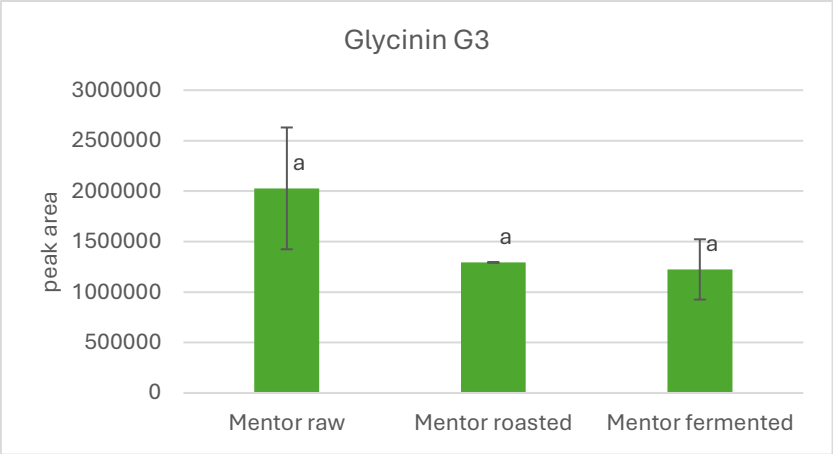

Figure 23: Relative comparison of the glycinin G3 content between raw, roasted and fermented Mentor samples; variables with same letter = no statistically significant difference (α = 0.05).

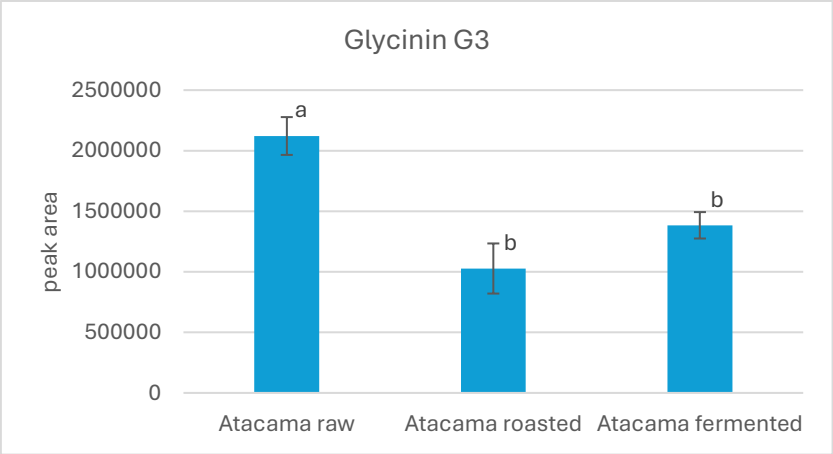

Figure 24: Relative comparison of the glycinin G3 content between raw, roasted and fermented Atacama samples; variables with same letter = no statistically significant difference (α = 0.05).

Although the height pattern of the bars in Figure 23 and Figure 24 appear similar, the SD of the raw sample in Figure 25 is smaller than in Figure 24. This lower variability resulted in the roasted and fermented samples being assigned to a statistically distinct groups, separate from the raw sample. The same pattern is observed in the varieties Lenka and Melanie, mirroring the results shown in Figure 24. This indicates a statistically significant reduction in the glycinin G3 content after both treatments in the roasted and fermented samples of all varieties but Mentor. Therefore, this protein seems to be less stable than glycinin G1 and G2, both of which suffered no losses after treatments in at least three varieties each. This is an unexpected result, as the glycinin subunits G1, G2 and G3 belong to the same group, share 80 % sequence identity and have shown very similar thermal stabilities[39]. It is possible that differences in the remaining 20 % of the sequence identity cause the G3 subunit to be more susceptible to roasting and fermentation than the other two. The glycinin G4 content also shows a decrease after roasting and/or fermentation, depending on the variety. Across all four varieties, the lowest amount of the glycinin G4 protein is consistently recorded in the fermented samples. The roasted samples of the varieties Atacama and Melanie also undergo a notable reduction,

although less pronounced in the fermented samples. In contrast, the varieties Lenka and Mentor have no clear statistical distinction between their raw and roasted samples. These results can be exemplified by Figure 25 for the variety Atacama and Figure 26 for Lenka.

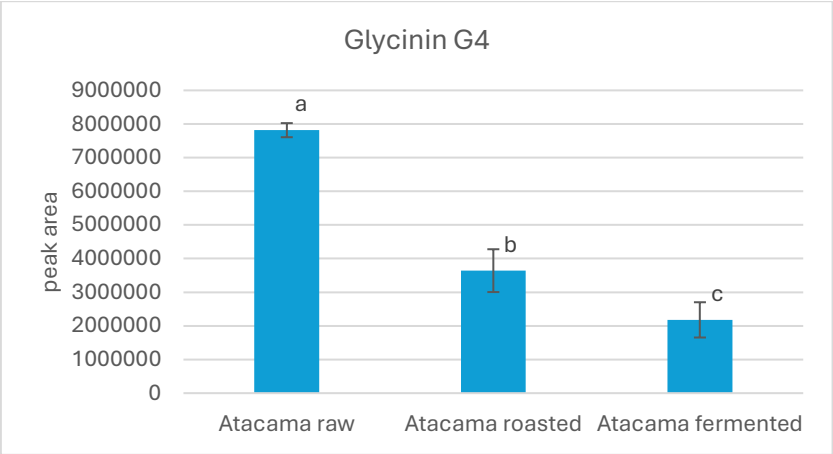

Figure 25: Relative comparison of the glycinin G4 content between raw, roasted and fermented Atacama samples; variables with same letter = no statistically significant difference (α = 0.05).

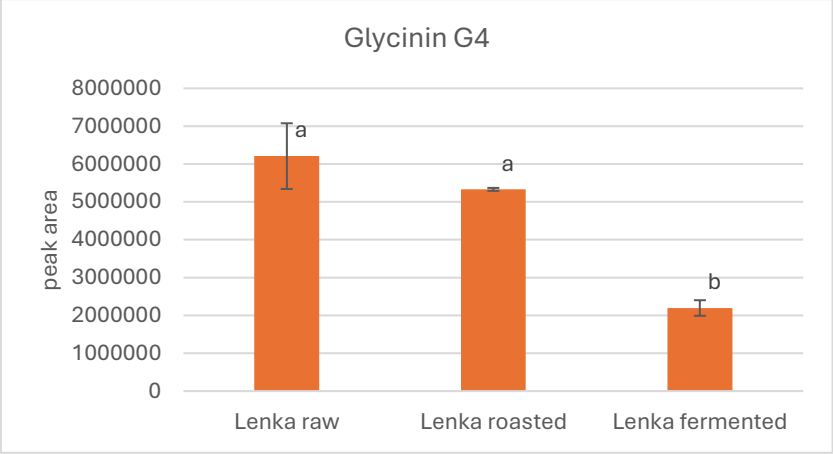

Figure 26: Relative comparison of the glycinin G4 content between raw, roasted and fermented Lenka samples; variables with same letter = no statistically significant difference (α = 0.05).

Glycinin G4 appears to be less stable under roasting and fermentation conditions, as evidenced by its reduced content in the roasted samples of two varieties and in the fermented samples of all four. These results may be caused by its lower sequence identity less than 50 % with glycinin G1 and G2, as it belongs to group II.[40] However, one source has reported the order of thermal stability as G1/G2/G3 < G5 < G4 (without its acidic polypeptide A4)[41] – this contradicts the results obtained within this work, as the content of the subunits G1 and G2 did not decrease as evidently as the subunit G4 did. Lastly in this protein series, the glycinin G5 content did not experience a notable decrease in the varieties Atacama and Melanie after either one of the treatments, seemingly stable towards heat and fermentation. However, the variety Lenka did suffer a statistically relevant reduction of the glycinin G5 amount in the roasted sample, as seen in Figure 27. Although the fermented sample also showed decrease, the reduction was not statistically significant.

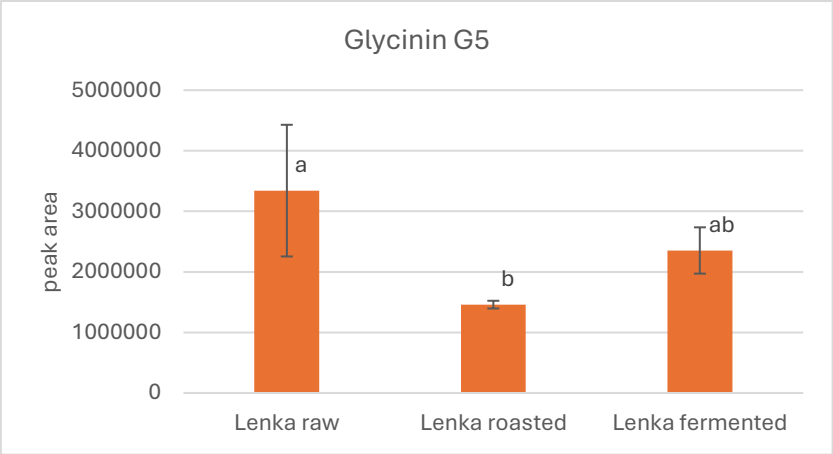

Figure 27: Relative comparison of the glycinin G5 content between raw, roasted and fermented Lenka samples; variables with same letter = no statistically significant difference (α = 0.05).

In Figure 28, the variety Mentor does not appear to have lost any glycinin G5 content. The fermented sample shows the highest amount, yet it remains within the same statistical group as the raw sample, indicating no significant difference from the initial amount. While one source has reported the G5 subunit to be the least thermally stable one[39], another source has reported it more thermally stable than the group I subunits.[41] Since three varieties showed no statistically significant reduction of glycinin G5, it can be assumed to be a rather stable protein comparable to glycinin G1 and G2.

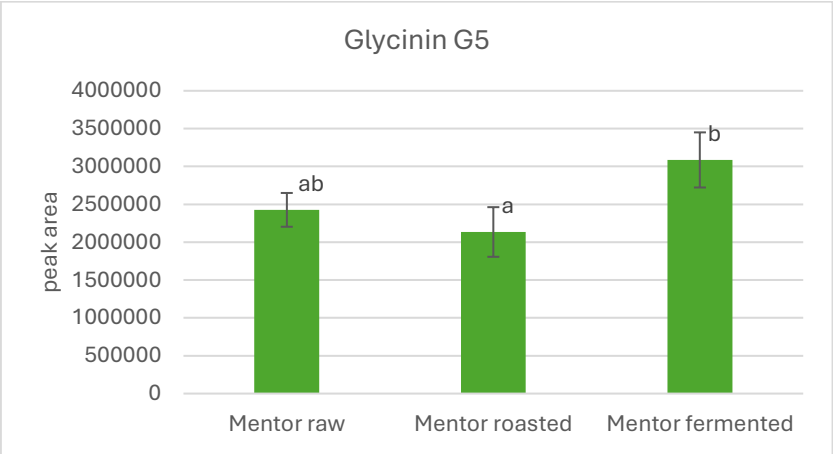

Figure 28: Relative comparison of the glycinin G5 content between raw, roasted and fermented Mentor samples; variables with same letter = no statistically significant difference (α = 0.05).

Thus, it can be concluded that three (G1, G2, G5) of the five glycinin subunits demonstrated relatively high stability under both heat-treatment and fermentation process, whereas the other two (G3, G4) were more susceptible to reduction. Although fermented samples exhibited slightly higher losses, particularly in the case of glycinin G4, there was no substantial difference between the two treatments overall. In contrast, a pronounced change was observed in the relative content of the β-conglycinin α protein after treatment. A sharp decline in the protein content is observed in the roasted samples, with even lower levels of the protein remaining in

the fermented ones. All four varieties display the same pattern, exemplified by the results of the variety Atacama in Figure 29.

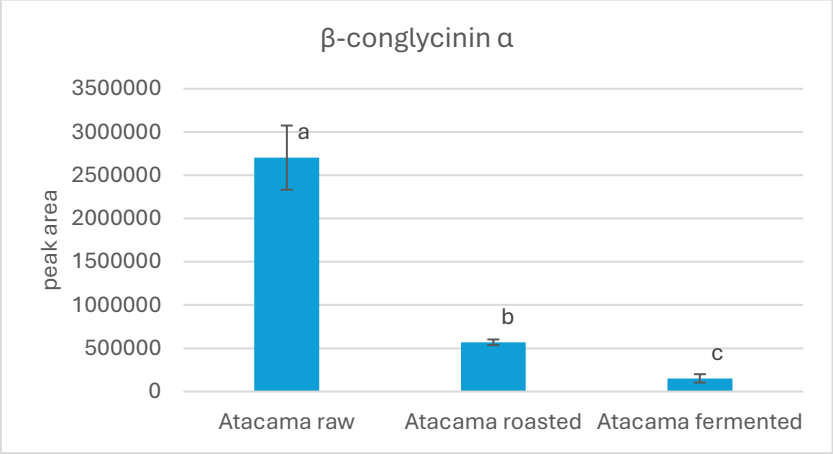

Figure 29: Relative comparison of the β-conglycinin α content between raw, roasted and fermented Atacama samples; variables with same letter = no statistically significant difference (α = 0.05).

A similar trend was detected in the β-conglycinin β content. In the varieties Atacama and Lenka, both treatments led to a significant loss, although with no statistically relevant difference between the roasted and fermented samples. The results for the variety Atacama can be seen in Figure 30 to serve as an example.

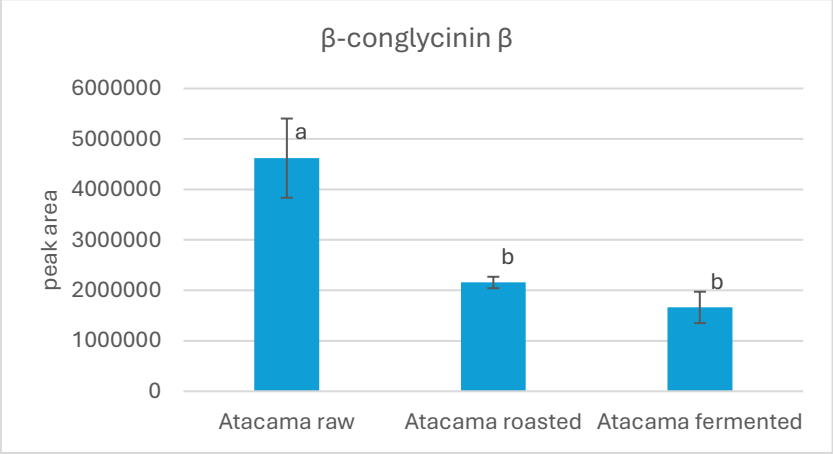

Figure 30: Relative comparison of the β-conglycinin β content between raw, roasted and fermented Atacama samples; variables with same letter = no statistically significant difference (α = 0.05).

The variety Melanie showed no statistically significant difference in the β-conglycinin β content after roasting. However, a significant reduction was observed after fermenting, as seen in Figure 31. Meanwhile, the variety Mentor did display a significant difference between the raw and fermented sample; the roasted sample displayed an intermediate value , placing it within both statistical groups, as shown in Figure 32.

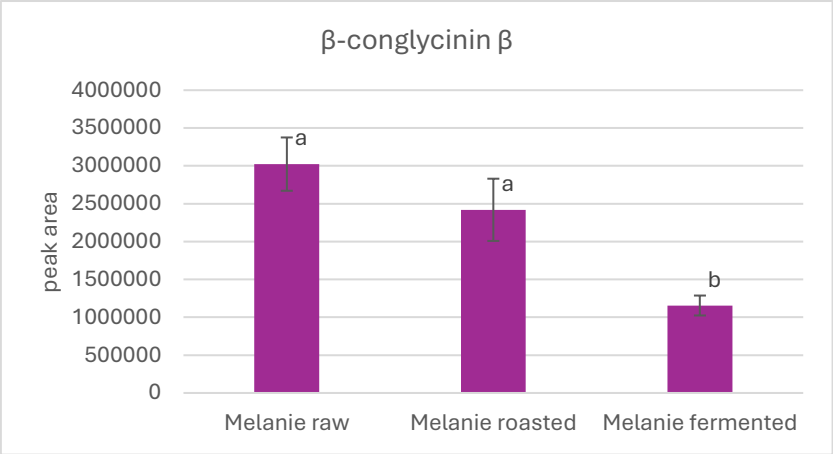

Figure 31: Relative comparison of the β-conglycinin β content between raw, roasted and fermented Melanie samples; variables with same letter = no statistically significant difference (α = 0.05).

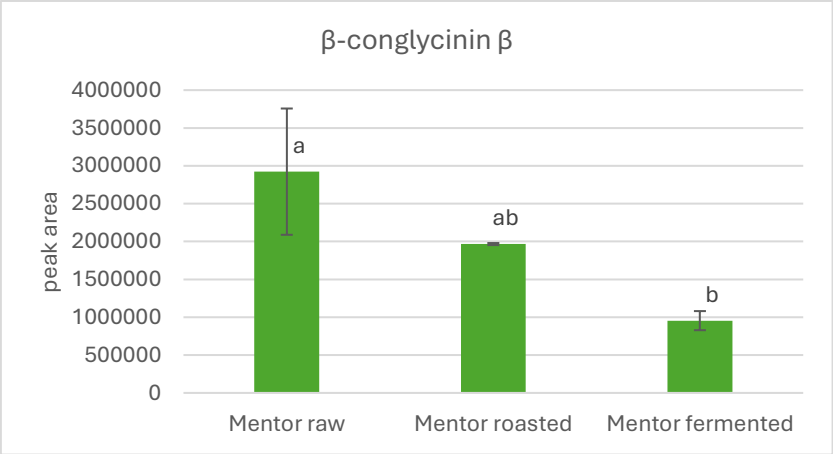

Figure 32: Relative comparison of the β-conglycinin β content between raw, roasted and fermented Mentor samples; variables with same letter = no statistically significant difference (α = 0.05).

According to these results, the β-conglycinin proteins seem to be rather labile, especially towards fermentation. In many cases, these proteins also exhibited losses after roasting. This is likely due to the β-conglycinin proteins lacking stabilising disulfide bonds in contrast to the glycinins.[5] The α subunit of β-conglycinin is reported to be less (thermally) stable than the β subunit[42], which aligns with the results seen in Figure 30, where the α subunit undergoes significant degradation after both treatments. Furthermore, fermentation utilizing *Lactobacillus plantarum* strains have been observed to degrade β-conglycinins, especially the α subunit.[32] This was clearly observed in Figure 30. Similarly, the basic 7S globulin proteins display high lability towards both treatments. The relative comparison of the basic 7S globulin 1 content for the variety Atacama is displayed in Figure 33, where the roasted sample shows a significant reduction and the fermented sample an even greater loss. Comparable results were also obtained for the variety Lenka

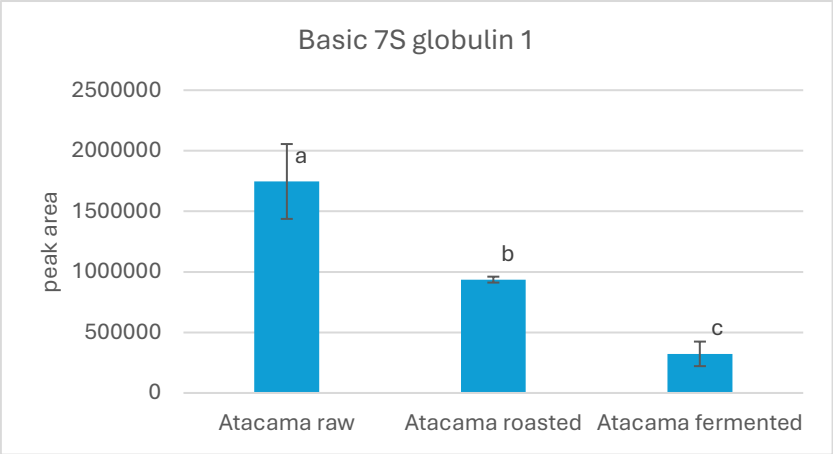

Figure 33: Relative comparison of the basic 7S globulin 1 content between raw, roasted and fermented Atacama samples; variables with same letter = no statistically significant difference (α = 0.05).

The 7S globulin 1 content of the roasted Melanie and Mentor samples were not significantly reduced compared to the initial amount in their respective raw samples, while the fermented ones were. The results of the variety Melanie can be seen in Figure 34.

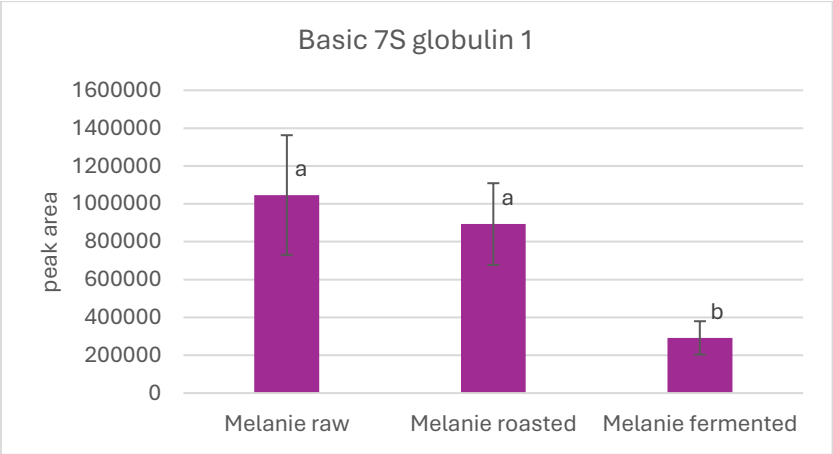

Figure 34: Relative comparison of the basic 7S globulin 1 content between raw, roasted and fermented Melanie samples; variables with same letter = no statistically significant difference (α = 0.05).

In the case of the basic 7S globulin 2 protein, a pronounced decrease is observed in the amount after both treatments. The roasted and fermented samples of the variety Atacama differ significantly from the raw sample, but not from each other. The variety Lenka displays the same behaviour, which can be seen in Figure 35.

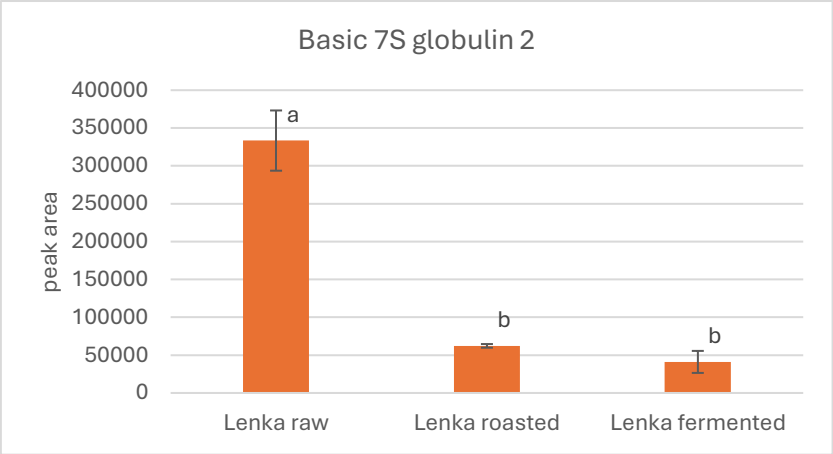

Figure 35: Relative comparison of the basic 7S globulin 2 content between raw, roasted and fermented Lenka samples; variables with same letter = no statistically significant difference (α = 0.05).

A statistically significant difference is observed between all samples of the variety Melanie, where the roasted sample is significantly reduced while the fermented sample displays the lowest amount. As the variety Mentor has comparable results, it can be used as an example, which can be seen in Figure 36.

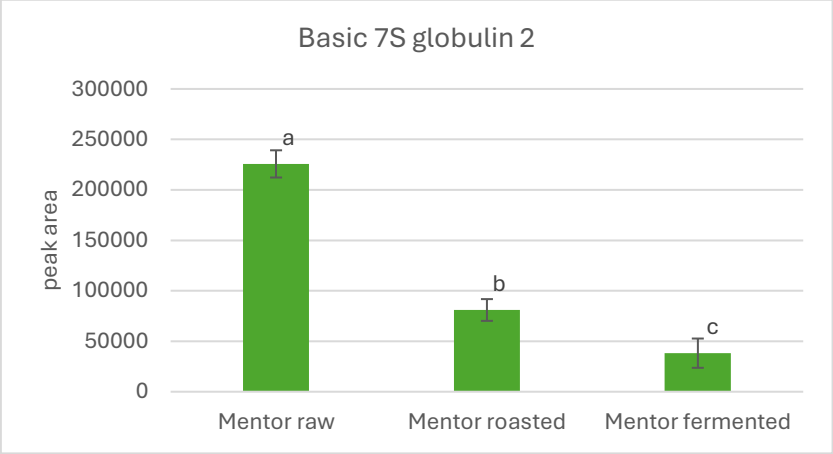

Figure 36: Relative comparison of the basic 7S globulin 2 content between raw, roasted and fermented Mentor samples; variables with same letter = no statistically significant difference (α = 0.05).

To sum up, the lowest level of the basic 7S globulins were most often observed in the fermented samples. The roasting process resulted in significantly reduced levels as well, although never to the extent detected after fermentation. The basic 7S globulins 1 and 2 share a sequence identity of 93 % and contain multiple stabilising disulphide bridges, contributing to their compact structure. Furthermore, they have been reported to be very resistant to enzymatic cleavage.[43] While the literature suggests a rather stable protein, the findings of this work suggest otherwise. One reason for this discrepancy could be the low abundance of the basic 7S globulins[44], as being close to the limit of detection reduces quantitative reliability[45]. Additionally, the quantification in this work was based on a limited number of peptides, especially in the case of the basic 7S globulin 2, for which only one unique peptide was used (Table 5). It is recommended to use multiple peptides per protein to ensure reliable quantification.[46] Peptides

containing oxidation-prone residues like methionine in the basic 7S globulin 1 protein (Table 5) may not be detected in their modified form, leading to an underestimation of the protein abundance.[47] These factors might explain the difference between the stability observed for these proteins within this work and the stability reported in literature.

Another case where both treatments affected the relative protein content similarly was the Kunitz-type trypsin inhibitor KTI1 in the varieties Atacama and Lenka. A statistically significant reduction was detected in their roasted and the fermented samples, with no difference between the two treated samples. As both varieties display the same pattern, the results of Atacama can be used as an example, which can be seen in Figure 37.

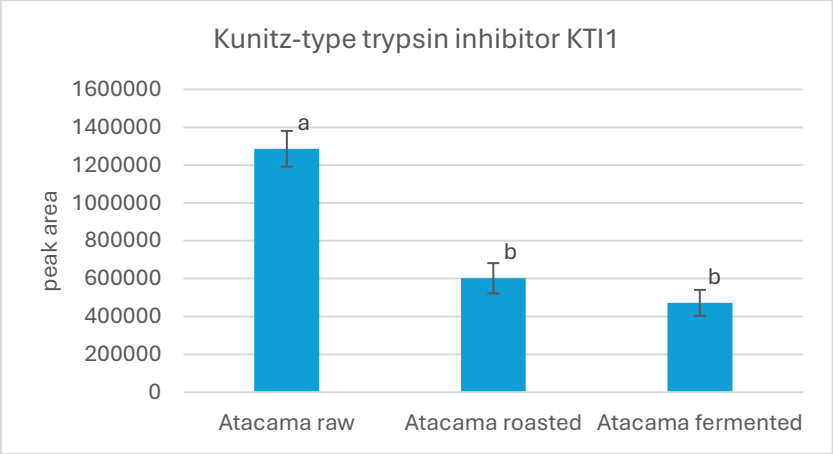

Figure 37: Relative comparison of the Kunitz-type trypsin inhibitor KTI1 content between raw, roasted and fermented Atacama samples; variables with same letter = no statistically significant difference (α = 0.05).

However, the varieties Melanie and Mentor did not exhibit a comparable degree of protein degradation. The results for the variety Mentor indicate a higher level of protein KTI1 in the roasted sample than in the raw sample, as seen in Figure 38. This could perhaps be the result of improved extractability or a concentration of the protein after sample treatment.[7] The fermented sample occupies an intermediate value, displaying no statistically distinct difference.

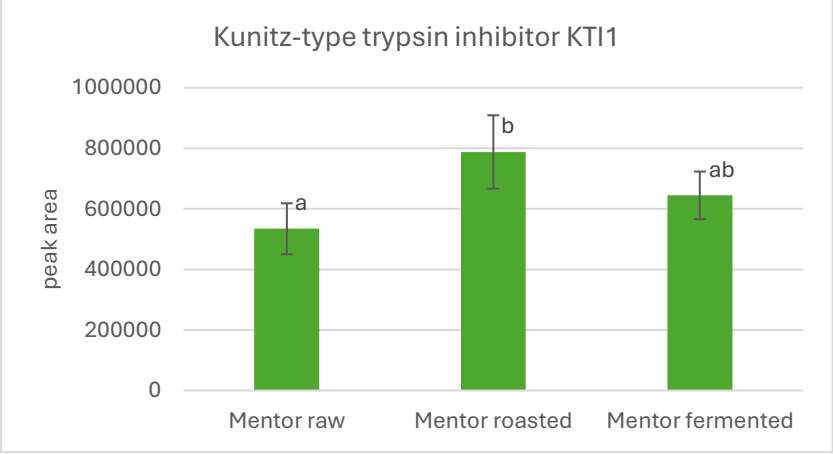

Figure 38: Relative comparison of the Kunitz-type trypsin inhibitor KTI1 content between raw, roasted and fermented Mentor samples; variables with same letter = no statistically significant difference (α = 0.05).

In the case of the variety Melanie the results of fermentation experiments could not be averaged per the statistical evaluation. While the first two fermentation experiments did not show a clear difference compared to the raw and roasted samples, the third fermentation experiment exhibited a noticeable reduction in protein content, seen in Figure 39.

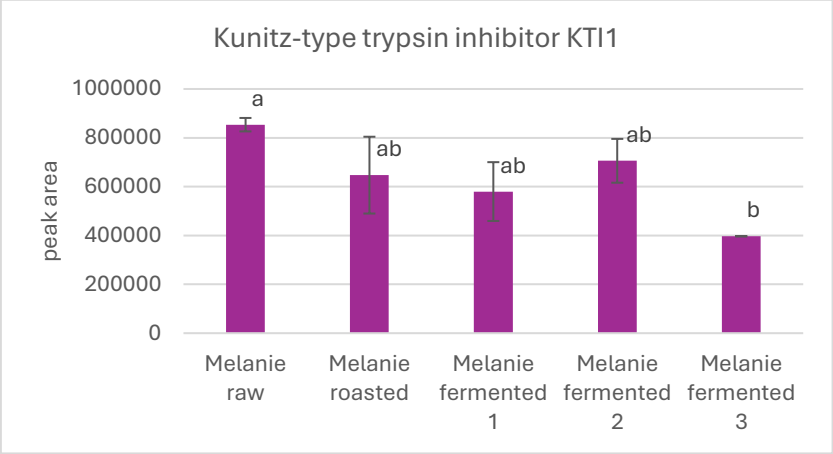

Figure 39: Relative comparison of the Kunitz-type trypsin inhibitor KTI1 content between raw, roasted and fermented Melanie samples; variables with same letter = no statistically significant difference (α = 0.05).

It is noteworthy that the varieties Melanie and Mentor contained the lowest amounts of KTI1 present in the respective raw beans compared to the other two varieties, as previously discussed in Figure 19. The amount of TIs in soybeans is relatively low, typically up to six %.[7] Samples with low concentrations may result in nonzero values below the limit of detection – excluding those nondetectable values can lead to highly biased estimations of the actual content.[48] Therefore, only the reliable results of the varieties Atacama and Lenka, which were the ones containing the highest initial amount of KTI1, are considered for the estimation of its stability. As a significant reduction of its content was recorded in at least two instances, this protein seems to be rather labile. This was expected, as TIs are generally considered labile proteins, especially KTIs due to only having two disulfide bonds.[11] A similar result was observed for the relative content of KTI3 in the variety Melanie, displayed in Figure 40, where the roasted sample seems to have a higher content than the initial amount in the raw bean. Perhaps a better extractability of the protein after roasting could have led to this result, or a concentration of the protein during sample treatment.[7] However, the wide range of the SD suggests that the variability of the data may be the most likely cause for the discrepancy.

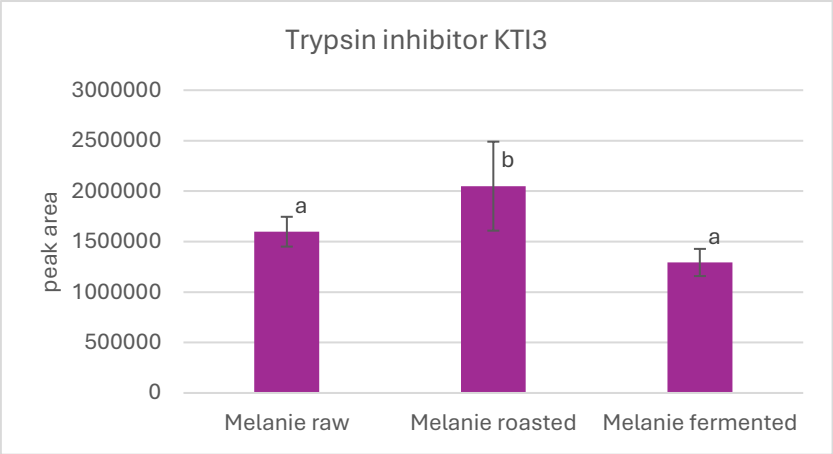

Figure 40: Relative comparison of the trypsin inhibitor KTI3 content between raw, roasted and fermented Melanie samples; variables with same letter = no statistically significant difference (α = 0.05).

The varieties Atacama and Mentor show no reduction of the KTI3 content after the treatments. On the other hand, the variety Lenka exhibited a significant loss under both treatments, as seen in Figure 41.

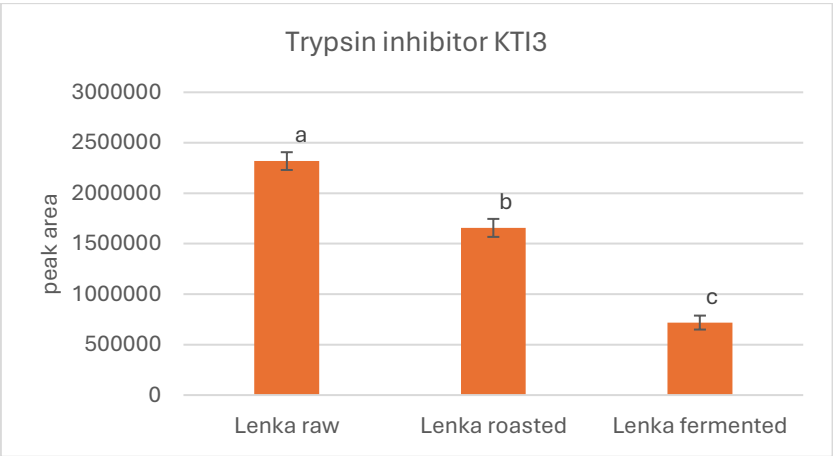

Figure 41: Relative comparison of the trypsin inhibitor KTI3 content between raw, roasted and fermented Lenka samples; variables with same letter = no statistically significant difference (α = 0.05).

According to the results in Figure 41, the protein KTI3 appears to be labile to both roasting and fermentation, with statistically significant differences observed. However, this lability was only observed for the variety Lenka. The other two varieties showed no statistically significant reduction, and one yielded unreliable results, making it difficult to conclude definitively about the stability of this particular subunit solely based on these findings. Literature suggests that KTI proteins are labile in general[11], which only the results from the variety Lenka corroborate. On the other hand, literature classifies Bowman-Birk trypsin inhibitors as relatively stable proteins compared to the KTI, as they possess seven disulfide bonds.[11] One representative protein of that class would be the Bowman-Birk type proteinase inhibitor C-II, or IBBC2 in short. Before evaluating the results, it must be mentioned that in some cases, some data points were missing for this protein. Normally, the results of three technical replicates are averaged per sample – provided no outlier is found according to the Dean-Dixon Q test – but some replicates

had no detectable IBBC2 content at all, most likely due to being below the limit of detection as BBI is found in smaller quantities than KTI[37]. Out of the four varieties, only the variety Atacama had no values missing in any sample replicates. Its results can be seen in Figure 42, where the initial amount in the raw Atacama sample seems to be lower than in the treated ones. Furthermore, no statistically significant difference between its roasted and fermented samples was recorded.

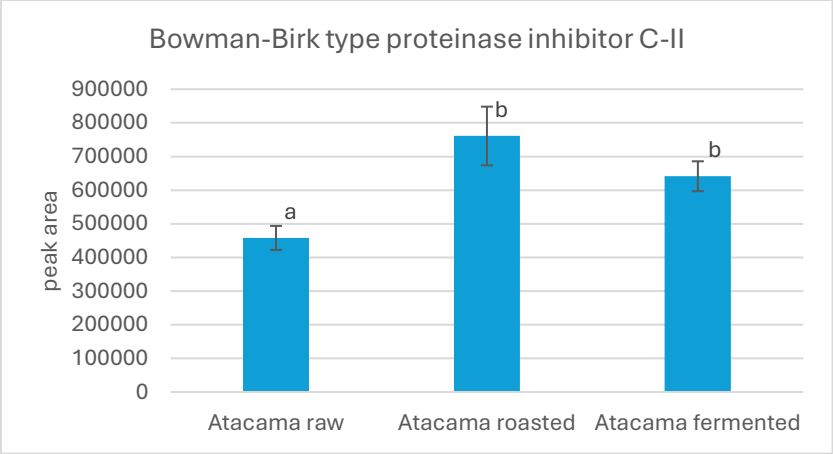

Figure 42: Relative comparison of the Bowman-Birk type proteinase inhibitor C-II content between raw, roasted and fermented Atacama samples; variables with same letter = no statistically significant difference (α = 0.05).

In contrast, the varieties Lenka, Melanie and Mentor exhibited pronounced SDs resulting in no statistically significant differences between any of their respective samples. As an example, the relative content of the IBBC2 in the variety Lenka can be seen in Figure 43.

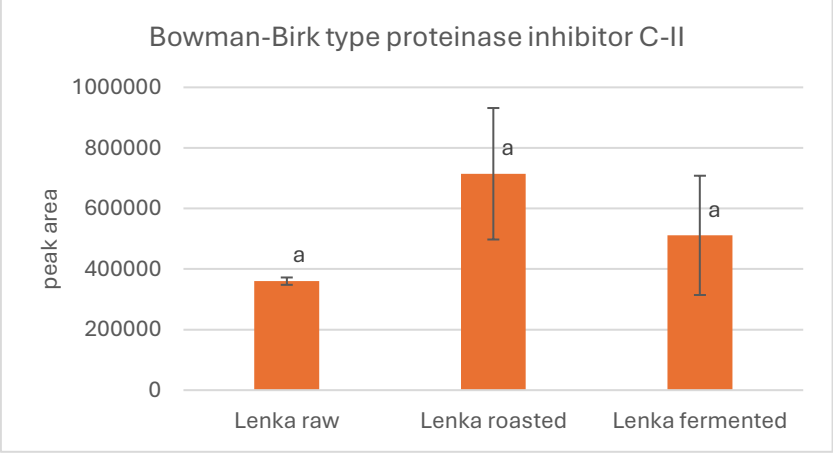

Figure 43: Relative comparison of the Bowman-Birk type proteinase inhibitor C-II content between raw, roasted and fermented Lenka samples; variables with same letter = no statistically significant difference (α = 0.05).

Based solely on the statistical evaluation, the samples of at least three varieties do not show statistically significant differences in the IBBC2 content after the treatments. However, the missing data points and therefore distorted results make this conclusion rather unreliable, as nondetectable values due to low abundance of the protein produce highly biased results like explained previously[48]. Similar results were observed in the case of the Bowman-Birk type proteinase inhibitor D-II, or IBBD2 in short. Although no data points were missing, for the variety

Atacama, the measurements are highly variable – presumably due to very low concentrations present in the samples. This leads to prominent SDs and consequently in statistically nonsignificant differences between its samples, as seen in Figure 44.

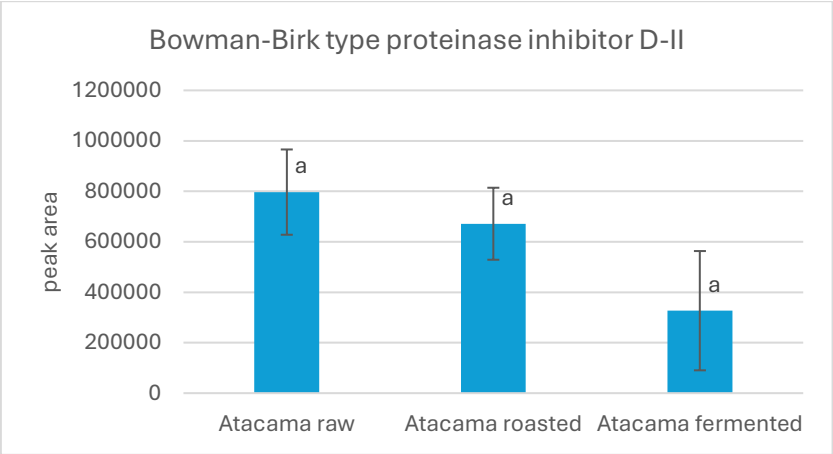

Figure 44: Relative comparison of the Bowman-Birk type proteinase inhibitor D-II content between raw, roasted and fermented Atacama samples; variables with same letter = no statistically significant difference (α = 0.05).

The remaining three varieties had missing data points for IBBD2, which limits the reliability of the statistical analysis. The results for the variety Lenka can be seen in Figure 45, where a significant difference in the IBBD2 content was recorded between the raw and treated samples. However, no significant difference is observed between the fermented and roasted samples of the variety Lenka

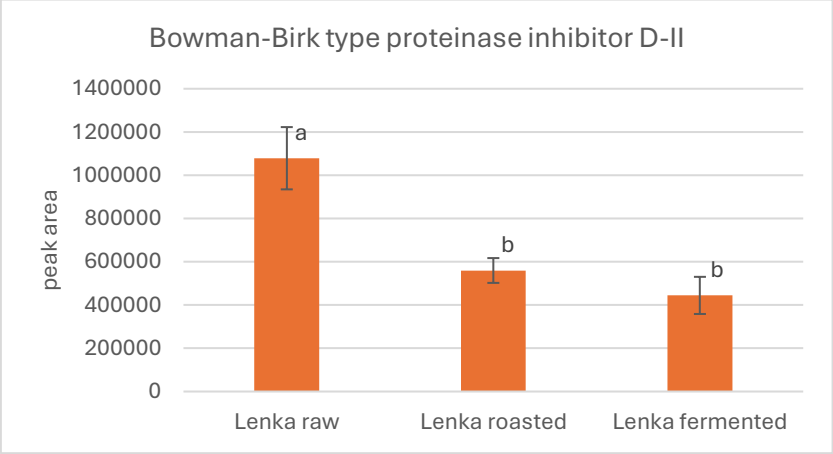

Figure 45: Relative comparison of the Bowman-Birk type proteinase inhibitor D-II content between raw, roasted and fermented Lenka samples; variables with same letter = no statistically significant difference (α = 0.05).

The variety Mentor displayed a statistically significant difference in the IBBD2 content between the raw and fermented sample, with the roasted sample showing an intermediate value, seen in Figure 46.

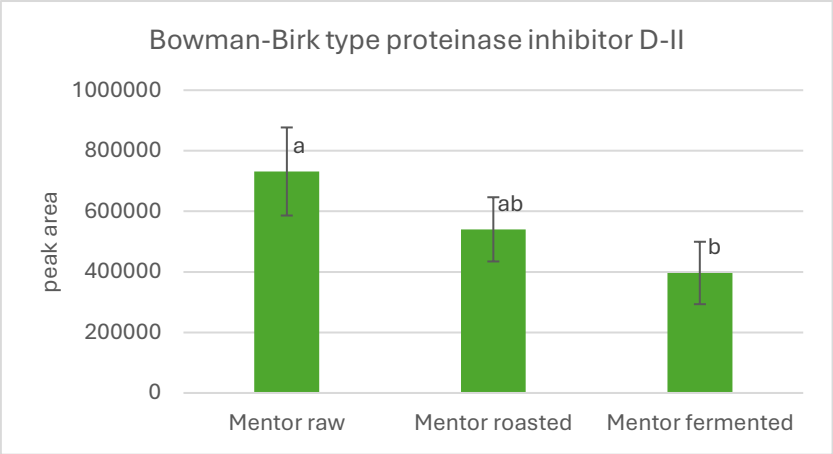

Figure 46: Relative comparison of the Bowman-Birk type proteinase inhibitor D-II content between raw, roasted and fermented Mentor samples; variables with same letter = no statistically significant difference (α = 0.05).

In the case of the variety Melanie, no IBBD2 was detected in the sample of the first fermentation experiment while the other two experiments provided widely different results, as seen in Figure 47. Due to this variability, no average could be calculated. There is no statistically significant difference between the raw and roasted samples and the sample from the second fermentation experiment. The third fermentation experiment, however, did show a statistically significant difference in the content. Fermentation is a complex biological process[4], which may have caused such highly varying results.

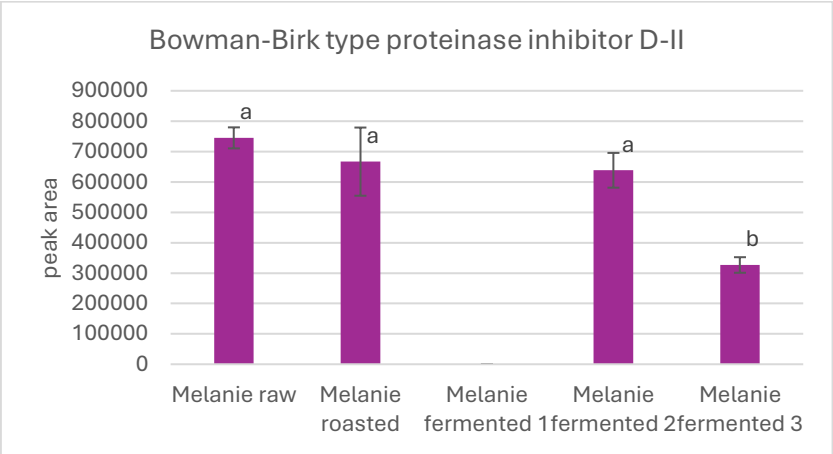

Figure 47: Relative comparison of the Bowman-Birk type proteinase inhibitor D-II content between raw, roasted and fermented Melanie samples; variables with same letter = no statistically significant difference (α = 0.05).

Due to the inconsistent results observed for the IBBD2 protein, no reliable conclusion can be made about its stability towards the treatments. As the TIs only make up a small part of the crude protein content[7] and the detected amount may be close to the limit of detection due to low abundance of the BBI[37], it is difficult to estimate how significant the loss of content really is. One protein where lability is most evident is β-amylase, which was detected in the raw samples only – it was absent in any of the treated samples across all varieties. These findings are consistent with literature reporting the stability of this protein declines rapidly upon heating, highlighting how labile β-amylase is to thermal treatment.[49]

It is important to reiterate that the high variability of the data must be considered when interpreting the results. The stability assessment is an estimation based on the statistical groupings afforded by ANOVA with post-hoc Tukey HSD test, which is a conservative test requiring a strong difference to declare significance[38].

3.5.3 Pearson correlation

A Pearson correlation was performed where the TIA values of the raw, roasted and fermented samples and the corresponding peak areas of the individual TIs resulted in the correlation coefficients listed in Table 7. Strong positive results (0.5-1.0)[50] are highlighted in green while moderate positive results (0.5-0.3)[50] are highlighted in yellow. Due to missing data in some samples, no average value could be calculated for the fermentation, hence the relationship between the TI amount and the TIA could not be determined. Such cases are marked with an "×".

Table 7: Pearson correlation coefficients for different trypsin inhibitors per variety; green = strong correlation; yellow = moderate correlation.

	KTI1	KTI3	IBBC2	IBBD2
Atacama	0.912	0.466	-0.992	0.489
Lenka	0.889	0.548	-0.971	0.858
Melanie	×	-0.556	-0.002	×
Mentor	-0.966	-0.810	×	0.712

According to literature, the inhibitory effect of KTI proteins is strongly related to their amount as they form a 1:1 stoichiometric complex with the active site of trypsin.[7] A strong positive correlation supporting this was observed for the KTI1 protein in the varieties Atacama and Lenka. However, the opposite trend was observed for the variety Mentor where a strong negative correlation coefficient would suggest that that the TIA is lower when KTI1 is more abundant. The results for the variety Mentor discussed in chapter 3.5.2 Figure 38 were not reliable, which likely caused this discrepancy. However, these correlation coefficients are non-significant as the p-value is larger than 0.05, likely due to the very small sample size reducing statistical power[51]. Thus, although two varieties scored strong correlation coefficients, a direct linear correlation of the KTI1 amount and the measured inhibition could not be statistically confirmed. While KTIs contribute up to 80 % to the TIA[11], a study has shown that the KTI3 subunit is responsible for most of the inhibitory activity in soybean[52]. A decrease of the KTI3 amount could therefore lower the TIA significantly. This was observed for the variety Lenka, which recorded a significant decrease of the KTI3 content after both treatments, discussed in chapter 3.5.2 Figure 41, and also a significant decrease of the TIA, discussed in chapter 3.4.

This resulted in a strong correlation coefficient. A drastic shift from positive to negative is observed in the next two varieties. The results for variety Melanie discussed in chapter 3.5.2 Figure 40 were rather unreliable while the variety Mentor did not record a statistically significant difference between any of its samples in chapter 3.5.2. Figure 41 – which likely led to the negative correlation coefficients suggesting an inverse relationship between abundance of the protein and the measured TIA. However, these correlation coefficients are not statistically significant either. The seemingly direct relation of the KTI3 protein and the TIA in two out of four varieties can therefore not be confirmed with statistical confidence.

The IBBC2 protein showed a strong negative correlation coefficient for the variety Atacama as well as Lenka, likely due to the results from chapter 3.5.2 being rather unreliable, seen in Figure 42 and Figure 43 respectively. Based on the correlation coefficient close to zero, the variety Melanie does not seem to exhibit any correlation between the IBBC2 content and the TIA, likely due to only exhibiting a statistically non-significant difference of the content after treatments, which was discussed in chapter 3.5.2. The IBBD2 protein appears to yield more consistent results across varieties, seen in chapter 3.5.2 Figure 44-Figure 47. While the coefficient for the variety Atacama suggests a moderate correlation, the varieties Lenka and Mentor show a strong positive correlation coefficient. But once again, none of these values are statistically significant. Thus, only a statistically non-significant correlation was found in three out of four varieties which would support the idea that a reduction of the inhibitor amount, in this case IBBD2, does lower the TIA.

As demonstrated in chapter 3.4, the most substantial loss in TIA occurred in the roasted samples across all varieties. In contrast, the relative content results presented in chapter 3.5.2 show variability. It is not always clear which treatment affected the TI content the most, if at all, not to mention the inconsistencies across the varieties. This means that despite a marked reduction in TIA, no corresponding loss in the TI content was recorded. Consequently, no clear correlation between inhibitory activity and inhibitor amount could be found in a consistent manner or in a statistically significant way within the varieties. It is also important to consider that the Pearson correlation only looks for linear relationships[50]. The lack of a (strong) correlation can mean multiple things. For example, for the cases where the TIA is very low but the content of a particular TI has not changed, the protein in question may not be a major contributor to the total trypsin inhibition detected. That was observed in the variety Melanie with the protein IBBC2 – while the roasted sample had a TIA below 4 mg/g, its IBBC2 content did not suffer a significant loss. It can be concluded that in general, both treatments rather inactivate the TIs than destroy them – roasting more so than fermentation.

4 Conclusion

Roasting of soybean meal resulted in negligible loss of the crude protein content, whereas fermentation caused a minor but statistically significant reduction. Both treatments led to significant reductions in soluble protein content, with fermentation causing slightly greater losses than roasting. Amino acid composition remained unchanged, although fermentation consistently resulted in small yet significant decreases in individual amino acids. Overall, differences between the two treatments in terms of protein quality were limited. In contrast, trypsin inhibitor activity was effectively reduced by roasting, with fermentation failing to lower the activity below the critical threshold of 4 mg/g for feed. Protein profiling by LC-MS revealed significant differences in only four proteins (glycinin G3, glycinin G4, β-conglycinin α, KTI1) between varieties, while the remaining proteins were comparable. Both roasting and fermentation led to a notable decrease of the abundance of several proteins (glycinin G3, glycinin G4, β-conglycinin α, β-conglycinin β, basic 7S globulin 1, basic 7S globulin 2), generally to a greater extent under fermentation, while some (glycinin G1, glycinin G2, glycinin G3) did not suffer a statistically significant loss after either one of the treatments. The response of the trypsin inhibitors (KTI1, KTI3, IBBC2, IBBD2) to the treatments was inconsistent, and no clear relationship could be established between trypsin inhibitor content and trypsin inhibitor activity. This suggests that fermentation inactivated rather than degraded the inhibitors. Taken together, these results indicate that the applied fermentation method is not a superior alternative to roasting for soybean processing. While fermentation reduced crude protein, soluble protein and amino acid content, it did not achieve the required reduction in trypsin inhibitor activity, which remains the most critical factor for improving soybean meal quality in animal feed.

5 Outlook

The fermentation method applied in this study did not reduce TIA to levels low enough to be considered a viable alternative to roasting in feed processing. However, it could be optimized in multiple aspects to potentially achieve lower TIA levels. The combination of lactic acid bacteria strains within the silage additive that was used for fermentation (Bonsilage) is originally formulated for grass fermentation. The strains could be tested individually or in different ratios to find a better composition suited for soybean fermentation, as previous studies[1] have demonstrated their effectiveness in reducing ANFs. Varying weight-to-volume ratios of the Bonsilage in the soybean samples could have been explored as well. Additionally, alternative fermentation approaches such as solid-state fermentation or a combination of solid and liquid could have been assessed. Fermenting techniques involving two steps with an aerobic and anaerobic stage have been effective according to literature[1] and could have been

evaluated using the Bonsilage. However, livestock is rarely fed soybean exclusively.[53] The fermented soy could be mixed into other feed ingredients in order to lower the TIA per unit of feed enough for safe consumption, as chronic intake of residual levels of ANFs in properly treated soybeans have been found to not be detrimental to the animal's health.[36] Additionally, the degradation of proteins with allergenic potential such as the subunits of the β-conglycinin protein[54] may be considered a beneficial outcome of fermentation. Furthermore, there are specialized cases where the lower protein solubility of the fermented samples may be beneficial, like in a ruminant diet.[14] In sum, many factors play into the nutritional value of soybean. The most prominent finding of this work is the isolated effect of fermentation, in contrast to many previous studies[25–27,32] that included a heated pre-treatment which may have influenced their results. While the present findings do not support fermentation as a direct alternative to roasting, several aspects remain unexplored. Fermentation may produce metabolites with beneficial bioactive properties that could offset the undesirable TIA effects. Moreover, other ANFs besides TIs, such as agglutinins or oligosaccharides, may be reduced more effectively by fermentation than by roasting, potentially yielding a feed ingredient superior to roasted soybeans. In summary, expanding the analytical approach beyond protein profiling could provide a more comprehensive understanding of the fermentation output. Given the potential, this fermentation method should not be abandoned but rather further investigated and optimized.

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7 Software and tools used

7.1 Reference management

Reference management was carried out with Mendeley Reference Manager (Version 2.136.0). All sources were cited using the American Chemical Society (ACS) format.

7.2 Writing assistance

The writing process was supported by AI through ChatGPT (GPT-4o, GPT-5). It assisted in improving grammar and style by suggesting alternative wordings to reduce repetitive phrasing. It was also employed to search for relevant publications.