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Effects of Storage Regimes on Chemistry, Functional Properties, and Vitamin Profile of Different Varieties of Chickpea

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EFFECTS OF STORAGE REGIMES ON CHEMISTRY, FUNCTIONAL PROPERTIES, AND VITAMIN PROFILE OF DIFFERENT VARIETIES OF CHICKPEA

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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Dedicated to my grandfather,

"**Habib Edrisi**"

I want your memory to always remain in my mind. Your departure became a turning point in my life.

You will always stay in my heart and mind.

With love,

Your first grandchild.

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ABSTRACT

EFFECTS OF STORAGE REGIMES ON CHEMISTRY, FUNCTIONAL PROPERTIES, AND VITAMIN PROFILE OF DIFFERENT VARIETIES OF CHICKPEA

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Chickpea (cicer arietinum) samples from five distinct varieties (crown, royal, orion, sierra, and frontier) were subjected to varying storage conditions to investigate the impact of temperature and relative humidity (rh) on their chemical, functional, physical properties, and vitamin b profile. over 360 days, samples were stored at two temperatures (21°c and 40°c) and three rh levels (40%, 55%, 65%), with collections made every 90 days. initial observations focused on key chemical properties including moisture, protein, fat, and total starch content. subsequently, protein and starch fractions were isolated for further analysis, employing techniques such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (sds-page) to identify protein bands, fourier transform infrared spectroscopy (ft-ir) to examine amid ii structures, and scanning electron microscopy (sem) to assess granular structure. additionally, functional properties such as pasting behavior, water holding capacity (whc), oil holding capacity (ohc), water solubility and absorption index (wsi and wai), as well as foaming and emulsion capacity (fc and ec) were evaluated. furthermore, the effects of storage conditions on the vitamin b profile were investigated, analyzing levels of thiamin (b_1), riboflavin (b_2), niacin (b_3), pyridoxin (b_6), and folic acid (b_9) using hplc-uv. while slight decreases were observed in fat, starch, and protein content, most varieties exhibited no significant ($p>0.05$) differences under harsh conditions (40°c and 65% rh). also, frontier protein content was significantly ($p\leq0.05$) the highest after 180 and 360 days

of storage that can be considered for protein isolation industry. while royal had the highest total starch content which significantly ($p \leq 0.05$) decreased after 180 and 360 days in 65% rh and 40°c condition, frontier had the lowest total starch content in 55% rh and 40 °c after 360 days. furthermore, functional properties such as whc changed significantly ($p \leq 0.05$) for orion and frontier after 180 days under 40°c but wai, wsi, fc, and ec changed significantly ($p \leq 0.05$) for all varieties for harsh conditions after 180 days. sds-page analysis indicated the disappearance of certain bands, while ft-ir results suggested increased aggregation and random coil content under harsh conditions for all varieties. the vitamin content and gel strength for all varieties showed a decline, whereas peak viscosities increased after 360 days of storage under harsh conditions (40°c and 65% rh). these findings underscore the importance of optimal storage conditions to preserve chickpeas' nutritional quality and functional properties over extended periods.

Keywords: chickpea, *cicer arietinum*, water-soluble vitamin, chickpea starch, protein functionality, storage

Chapter one

Chickpea starch and protein properties: a review of functionality, chemistry, isolation, and analytical methods

1. Abstract

Chickpea (*Cicer arietinum*) is one of the most important legumes in semiarid regions annually. The climate changes and growing population have challenged food production and food security recently. Plant-based proteins and by-products from pulse provide sustainable sources of high value. Chickpea has a high potential for use in different food applications due to the high protein (15-23%), high amylose (40-60%) in starch, and fiber (18-22%) content present in the flour. Many studies have been conducted to evaluate starch and protein structure in different types and cultivars of chickpeas, and diverse situations of storage to understand the functionality of protein and starch in food applications. This literature provides an overview of chickpea composition, protein, and starch effectiveness in food applications, potential applications, and new trends such as gluten-free and dairy-free programs. Before knowing the structure and functionality of the chickpea protein and starch, the isolation of protein and starch plays an important role due to its effects on product quality. Isolation has two important main methods, dry and wet fractionation. The focus is on wet fractionation because it results in high purity of starch and protein. Important methods were reviewed to evaluate protein and starch structure, reactions, quantification, and qualification in food samples.

Keywords: chickpea, *Cicer arietinum*, plant-based protein, chickpea starch, protein functionality

1.1. Introduction

According to the Food and Agriculture Organization, 10 primary and five minor pulses are cultivated all around the world. Chickpeas (*Cicer arietinum* L.), lentils (*Lents culinaris* Medik), peas (*Pisum sativum* L.), and bitter vetch (*Vicia ervilia* [L.] Willd.) are the most important pulses introduced as part of sustainable food production. These crops are nutrient-rich and have been cultivated since early farming communities (FAO, 2016).

Chickpea is a member of the *Papilionoid* subfamily of legumes and is the second-most worldwide cultivated annual legume crop after soybean. Chickpea cultivation is common in the Mediterranean basin, India, Pakistan, Mexico, Ethiopia, Southern Europe, Northern Africa, and North and South America and, it is principally cultivated in Mediterranean countries since it is adapted to semi-arid climates. World dry chickpea production was 15,871,846 tons in 2021 while India produced 7,118,491 tons of total world production and cultivation area of 15.004,885 ha by 2021 and Asia produced about 84.9% of the world between 1994 to 2021 (*FAOSTAT,* 2021).

There are two main varieties of chickpeas (*Cicer arietinum* L.) Kabuli and Desi types. The Kabuli type (Mediterranean and Near-East region has a light seed and the Desi type grown mainly in the Indian subcontinent and East Africa) has smaller and yellow-brown-colored seeds. The test weight and 1000 seed weight of Kabuli chickpeas ranged from 58.2-66.5 (lb/Bu) and 294-578 g respectively (Pulse Survey, 2021).

Due to the increasing demand for plant-based protein production, chickpea have a high potential to be incorporated into many food applications, for example, plant-based burgers, and the production of gluten-free high-protein content bread (Zhao et al., 2021).

Partially replacement of chickpea flour with wheat flour, increases resistant starch, fiber, and mineral content while increasing the gelatinization onset temperature and decreasing its enthalpy of gelatinization and viscosity (Lu et al., 2022). In addition to nutrient composition, chickpea has many health benefits such as decreasing type-2 diabetes and cardiovascular disease (Saget et al., 2020).

Although chickpea have been associated with positive health outcomes, only the chemistry, processing, and food application will be highlighted. Overall, the information in this review is focused on protein and starch isolation, composition, and functionality.

1.2. General Composition Overview

Chickpea flour has higher protein, fat, ash, and fiber content compared to wheat flour. Examples of the variability in composition among chickpeas are provided in Table 1.1. However, among pulses, chickpeas have a relatively high fat content (4-8%). The fatty acids composed in triacylglycerols include polyunsaturated, monounsaturated, and saturated, respectively (Das et al., 2020).

In addition to fat, total dietary fiber accounts for 19.5%-25% of the seed weight and is comprised of 5%-12% soluble fiber and 15%-22% insoluble fiber (Kaur & Prasad, 2021). Chickpeas are a significant source of micronutrients such as B vitamins and several microminerals like copper, iron, and zinc. Folate is the best example of a B vitamin. There is approximately 351-589 µg folate/100 g chickpea and a 1 cup serving (35 g) of cooked chickpea can provide 41% of the daily recommended value (400 µg/100g). Minerals such as potassium (11.13±1.24-12.10 g/kg dry matter), magnesium (1.78±0.01-2.20 g/kg dry matter), calcium (1.50±0.00-1.69±0.19 g/kg dry matter), and phosphorus (3.42±0.67-

3.90±0.01 g/kg dry matter) account for most of the minerals in chickpea (Bampidis & Christodoulou, 2011). Microminerals such as magnesium, iron, selenium, and zinc also contribute to health benefits (S. Xiao et al., 2023).

Table 1.1. The approximate composition of Kabuli and Desi types of chickpeas.

Composition (%)	Kabuli	Desi
Protein	15.2-25.3	19.3-22.50
Fat	4.0-9.35	6.35-6.65
PUFA[a]	49.34-57.77	55.94-58.20
MUFA[b]	25.72-36.78	27.12-28.66
SFA[c]	13.88-16.51	14.67-15.41
Total carbohydrate	67.6	48.2
Total starch	34.6-48.8	27.15-39.1
Dietary fiber	22.25	18.70
Insoluble fiber	12.50	10.70
Soluble fiber	9.75	8.00
Ash	2.0-4.3	2.59-2.66

[a]Poly Unsaturated Fatty Acid
[b]Mono Unsaturated Fatty Acid
[c]Saturated Fatty Acid

1.2.1. Protein and protein fractions

The protein of chickpeas ranges from 15.2% to 25.3% in Kabuli and from 19.3% to 22.5% in the Desi type. They contain essential amino acids (Table 1.2) such as methionine, threonine, histidine, valine, phenylalanine, isoleucine, tryptophan, lysine, and leucine, and essential unsaturated fatty acids such as linoleic acid (Ani & Thabit, 2021). Three major proteins of chickpeas include albumin (8%-12%), globulin (53%-60%), prolamin (3%-7%), and glutelin (19%-25%) (Eze et al., 2022). The whole molecular weight of chickpea protein is about 320-400 kDa. Moreover, 32- and 22 kDa subunits appear upon reducing conditions used in SDS-PAGE. The overall amino acid profile (Table 1.2) indicates that chickpea tends to have more essential amino acids than wheat and pulses such as pea and lentil. According to the amino acid profile (Table 1.2), chickpea has a higher amount of essential amino acids (39.89 g/16g N) compared to lentil, pea, and wheat.

For example, chickpea has 3 times higher lysine 6 (g/16g N) content compared to wheat (2.14 g/16g N) and lower than pea (7.6 g/16g N) and lentil (6.7 g/16g N). Phenylalanine content in chickpea is about 5.57 (g/16g N) which is higher compared to wheat (4.48 g/16g N), pea (4.8 g/16g N), and lentil (5 g/16g N). Also, chickpea is high in non-essential amino acids (98.53 g/16g N) (Rachwa-Rosiak et al., 2015).

Table 1.2. Amino acid profile (g/16g N) of peas, lentils, and chickpeas compared to wheat (Khazaei et al., 2019; Rachwa-Rosiak et al., 2015).

Type of amino acid	Wheat	Pea	Lentil	Chickpea
		Essential-amino acids		
Leucine	6.96	7.3	7.2	7.59
Isoleucine	4.25	4.1	4.1	4.76
Lysine	2.14	7.6	6.7	6
Methionine	2	1	0.9	1.54
Cysteine	1.33	1.3	1.1	1.36
Phenylalanine	4.48	4.8	5	5.57
Tyrosine	3.5	3.3	2.5	3.58
Threonine	2.6	3.8	3.7	3.86
Valine	4.94	4.5	4.7	5.6
Essential amino acids	32.2	37.7	35.9	39.89
		Non-essential amino acids		
Alanine	3.94	4.3	4.2	4.88
Arginine	3.61	8.2	7.8	7.82
Aspartic acid	4.64	11.3	10.7	11.18
Glutamic acid	26.59	16.4	16.1	18.05
Glycine	3.36	4.3	4.1	4.3
Histidine	2.45	2.3	2.4	2.96
Proline	8.11	4.4	3.8	4.68
Serine	3.85	4.9	4.7	4.77
Non-essential amino acids	56.55	56.1	53.8	58.64
The total amino acids	88.75	93.8	89.7	98.53

Generally, the sulfur-containing amino acids are higher in wheat than in chickpeas. In recent years many studies have been conducted to examine the protein antinutritional compounds and their effects on the reduction of certain forms of cancer, obesity, and the induction of innate defense mechanisms. Antinutritional protein compounds include

lectins, protease inhibitors, and the non-antinutritional component, and angiotensin I-converting enzyme (ACE) inhibitors (Roy et al., 2010).

1.2.2. Protein Quality and Digestibility

The Protein Digestibility-Corrected Amino Acid Score (PDCAAS) is a method used to evaluate protein quality by considering both its amino acid composition and digestibility. The PDCAAS of chickpea protein is typically reported to be around 0.76 (76%). This score suggests that chickpea protein is a good source of essential amino acids but may have certain limitations that prevent it from fully meeting the nutritional requirements of humans. It is important to note that PDCAAS values are not fixed and can vary depending on factors such as processing methods and the specific variety of chickpeas used. These limitations result in a reduction in protein digestibility and lower nutritional value, compounded by the existence of anti-nutritive factors (Nosworthy et al., 2020). Consequently, various processing methods such as baking, cooking, germination, and extrusion have been employed to mitigate the activity of anti-nutritional factors and enhance the bioavailability of the protein. For instance, it has been demonstrated that fermentation leads to an increase in essential amino acids, total sulfur, total aromatic compounds, and threonine(Angulo-Bejarano et al., 2008).

To accurately assess the protein quality and nutritional value of chickpea protein, the calculation of PDCAAS necessitates the establishment of a significant correlation between in vivo and in vitro methodologies. This correlation is vital to identify the most appropriate scoring methodology (Nosworthy et al., 2020). According to previous research on chickpea-isolated protein (Tavano et al., 2016), Chickpea flour exhibits suboptimal

amino acid scores for Valine (0.97), Methionine + Cysteine (0.75), and Tryptophan (0.84). Conversely, the albumin fraction derived from the same source has a complete score for all amino acids except Tryptophan (0.94). Globulin and glutelin fractions have limitations in Methionine + Tryptophan amino acids (0.72 and 0.38, respectively).

1.2.3. Globulin and Glutelin

Salt-soluble proteins called globulins account for 50% of the chickpea protein, which is further grouped as 11S (legumin), 7S (vicilin) at a ratio of 4-6:1, and 7S (convicilin). The 7S protein in chickpeas is a storage protein comprising a series of polypeptide fragments with various molecular weights (Chang et al., 2022). Legumin (11S, between 360 and 400 kDa) is an oligomeric protein and hydrophobic with six quaternary monomer subunits that are linked by a disulfide bridge alongside an acidic chain sited at the surface and a hydrophobic basic unit attached inside. Legumin has a higher amount of sulfur-containing amino acids than vicilin. Under reducing conditions, the SS linkage is broken. the acidic subunit of legumin-α can be detected at ~40 kDa and a basic subunit of legumin-β at ~20 kDa. Vicilin (160–200 kDa) has no cysteine and is not reduced due to a lack of the SS linkage showing bands between 10-20 kDa (Chang et al., 2022). Vicilin is a salt solution soluble (80% solubility at 0.2 mol/L NaCl) protein and has minimum solubility at pH 4.5 to 6.0. Instead, vicilin monomers can form non-covalent hydrophobic bonds that help to maintain tertiary structure (Shevkani et al., 2019).

Globulin and glutelin are considered less susceptible to proteolysis and affect pasting and textural parameters. Sodium chloride is a strong solubilizer for globulins in

legume seeds and potassium sulfate is a precipitant for them. In the presence of sodium chloride, most of the globulins behave like albumin (Liu et al., 2008).

1.2.4. Albumin

Albumin fraction in chickpea protein is present in the cytoplasm and is approximately 15-20% of cotyledonary proteins. In contrast to 7S and 11S storage proteins, albumins have an essential role in enzymatic and metabolic functions within the seed. For example, a trypsin inhibitor is an albumin that functions to inhibit protein digestion as well as being insecticidal (Kou et al., 2013). Albumins are water soluble and are a source of sulfur-containing (cysteine and methionine) essential amino acids as well as tryptophan, threonine, and lysine. In addition to being a source of essential amino acids, albumin has a very good foaming capacity (Ghumman et al., 2016).

1.3. Anti-nutritional factors

Inhibitors of amylase (proteins and peptides) and trypsin are the antinutritional factors that prohibit reaching pancreatic and amylase enzymes. α-Amylase is important in plants to reduce available starch and prevent organisms that can metabolite starch (Westermann & Craik, 2010). Trypsin inhibitor is mainly concentrated in the cotyledon (77-76%), embryonic axis (12-15%), and haul (11-9%) in protein content, cell walls, and cytosol. The total inhibitor activity of trypsin is 8-15.7 (U/mg). In this regard, many physical and chemical methods have been applied for the deactivation of trypsin, for example, one of the simple methods is boiling the seeds in water for 300 seconds completely inactivates the trypsin inhibitor (Avilés-Gaxiola et al., 2018; Márquez & Alonso, 1999).

Presence of the polyphenols such as tannins in chickpea is to bind to proteins with non-covalent interaction leads to a decrease in bioavailability and causes browning due to the polymerization of low molecular polyphenols to high molecular polyphenols which are brown colored (Mondor et al., 2009). The average of the tannin which was found among the two hundred and fifteen mini-core accessions of chickpea was about 56.64 mg/g (maximum was 189.63 ± 1.17 mg/g and the minimum was 0.232 ± 0.01 mg/g). Also, the average amount of phytic acid was 0.91 mg/g (the maximum was 4.06 ± 0.05 mg/g and the minimum was 0.009 ± 0.0 mg/g) (Bhagyawant et al., 2018). tannins can bind to the saliva proteins and digestive system mucosal proteins causing a reduction of the digestibility of proteins and carbohydrates. Moreover, tannins make resistance to enzymes of the digestive system leading to not absorbing nutrients.

1.4. Starch

Starch structures in "Kabuli" and "Desi" types are different. Kabuli type has higher amylose content compared to the Desi type and amylopectin of both is higher than amylose. Starch granules of chickpea are mainly composed of glucans, which are amylose (mainly large linear molecules). Amylose is a linear polymer of glucose molecules linked by α(1→4) glycosidic bonds. It typically constitutes about 20-30% of starch and forms a helical structure. Amylopectin is a branched polymer of glucose units, with α(1→4) glycosidic bonds forming the main chain and α(1→6) glycosidic bonds forming branches. Amylopectin accounts for about 70-80% of starch and has a highly branched structure and these two important parts are approximately 99% of dry basis, and the remaining comprises lipids, phosphates, and minerals esterified to hydroxyl groups (Zhang et al., 2019a). The

granular starch structure of chickpeas is mainly type-C based on crystalline structure. Starch fractions in chickpeas are categorized as native starch, resistant starch I, and resistant starch II. These fractions are obtained through multiple washing and precipitation steps and are comprised of small and large linear molecules in size of 221 to 579 kDa for amylose and 635,103 to 1160,103 kDa for amylopectin (Xu et al., 2013). The molecular structure of the starch is highly functional and are responsible for the physical and functional properties regarding gel strength, crystallinity, and digestibility (Sun et al., 2018).

The chemistry, structure, and morphological attributes of starch are responsible for gelatinization, swelling, solubility, gelation, transmission, and pasting properties (Wani et al., 2016). It was found that total protein content in chickpeas is significantly positively correlated with amylopectin molecular size and had a negative correlation with the amount of short-chained amylopectin (Tan et al., 2021).

1.4.1. Resistant starch

Resistant starch is one of the important parts of starch that cannot be digested by the small intestine of humans and remains undigested. Resistant starch in the colon can be fermented by microorganisms and produce short-chain fatty acids. On the other hand, resistant starch has many health benefits such as prebiotic properties and beneficial effects on body weight, glucose, and lipid metabolism. The resistant starch which cannot be digested, is classified as fiber (Bendiks et al., 2020; Liu et al., 2020).

It is assumed that starch should be digested completely in small intestines, but some researchers indicated that some parts of starch cannot be hydrolyzed completely

(Hasjim et al., 2013). Starches were divided into three categories, rapidly digestible starch, slowly digestible starch, and resistant starch. Resistant starch is divided into 5 groups itself. Resistant starch I (RSI) is the type that cannot be digested due to its structure on cell wall barriers in different whole grains such as legumes, resistance starch II (RSII) is known as crude starch and has a crystalline form that can be found in raw potato and green banana, resistance starch III which retrograded starch like cooked starchy food then cooling down and it has long branched chains of the amylopectin with double helices, resistance starch IV which is modified from original starch with the addition of the functional groups, and resistance starch V which comprises long chain unbranched starch with free fatty acids and form double helices leads to impossible to digest (Hasjim et al., 2013). The resistant starch fractions can be separated from native starch using wet fractionation (Sun et al., 2018). In this method, native starch was autoclaved and cooled down then amylase digestion was applied to isolate RSI. RSI was heated and again stable alpha-amylase was added to isolate RSII then to obtain RSIII, and RSIV, high-concentration K_2CO_3 solution was added and two components of floated matter and settled matter were extracted using washing steps, centrifugation, and freeze-dried. To separate amylose and amylopectin fractions from native starch, defatted and deproteinized starch was dissolved in 0.5 M warm NaOH, and neutral pH was adjusted using HCl. Then, 1-butanol and isoamyl alcohol were added and boiled for 20 min. Amylose precipitated after 4 hours of cooling and again after amylose separation from the solution, a latex-like supernatant was collected. These steps were repeated 6 times for amylose and amylopectin isolation to get pure fractions and dried.

1.5. Fractionation of flour

Before extraction, the chickpea can be subjected to different processes such as dehulling, soaking, or milling. These processes facilitate the penetration of water through the material composed of starch and protein (Boukid, 2021). Chemical extraction methods are based on the solubility of protein in alkali, acid, or organic solvents (Table 1.3). The chemical extraction process is considered mainly in three steps: defatting using n-hexane and n-pentane, extraction using salts such as NaCl, ionic detergents, and non-ionic detergents, and precipitation. However, commercial isolation of protein differs from laboratory approaches and results in less pure protein fractions. The preparation of protein and starch fractions can be achieved by dry and wet fraction processes. However, wet processes are more common because you can achieve higher purity.

Table 1.3. Solubility of protein fractions of legumes in different extraction solvents (Eze et al., 2022).

Solvents	Glutelin	Prolamin	Albumin	globulin	protamine
Water	-	-	+	-	+
Salt	-	-	+	+	-
Acid/alkali	+	-	-	-	+
Alcohol	-	+	-	-	-

-Means fraction is not soluble in the solution.
+Means fraction is soluble in the solution.

The chickpea flour ingredients include proteins, starches and fiber that are obtained through wet and dry fractionation processes (Figure 1). Wet fractionation is the main method to produce protein and starch fractions. Different methods of wet fractionation exist; however, all approaches can separate different protein and different starch components to some degree.

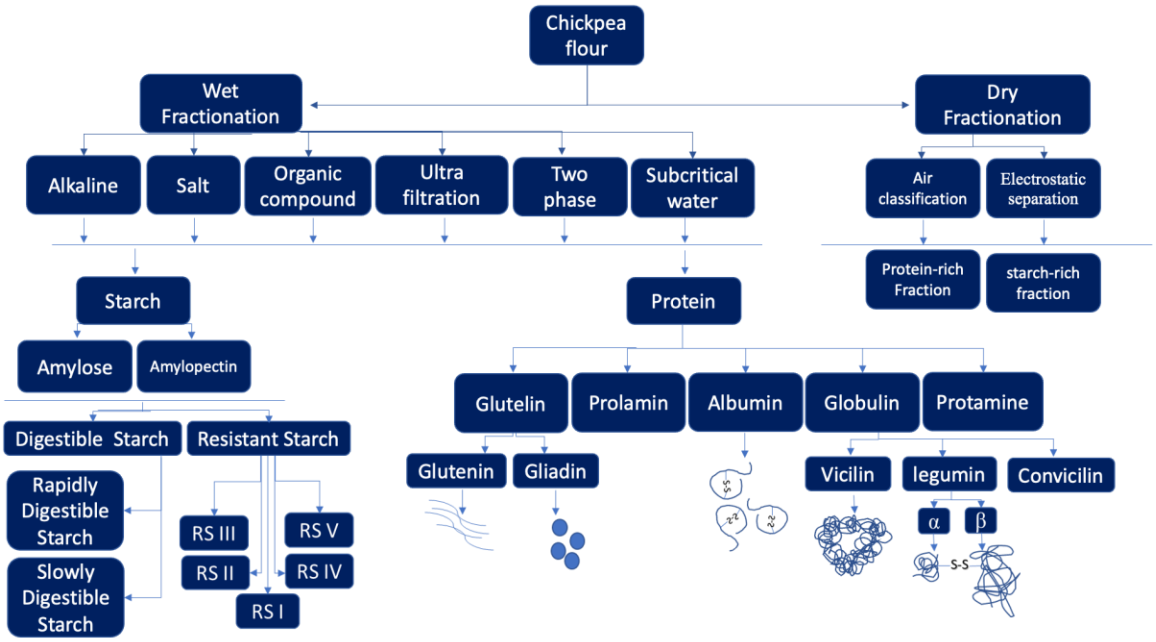

Figure 1.1. Flow chart depicting chickpea starch and protein production, fractions, and structure.

1.5.1. Dry fractionation

Starch isolation can be done using wet and dry processing approaches designed to separate starch and protein. Dry fractionation via milling (ball mills, hammer mills, and pin mills) uses less energy and produces no waste while functional properties of native protein are preserved (Pelgrom et al., 2015). Dry fractionation is based on particle size and weight to separate fractions using sieving, air classification, gravity separation, and magnetic separation. For example, protein particles are larger than starch and cannot go through the particular mesh size, and also, protein fractions are denser than starch-rich fractions and can be separated using gravity separation. Dry fractionation of chickpeas could increase protein content from 21.6 g/100g to 45.3 g/100g in protein-enriched flour (Pelgrom et al., 2015).

Dry fractionation or fractionation approaches produce less pure starch due to the lower capacity of air classification compared to wet fractionation, but the use of dry fractionation to produce protein-rich coproduct could be the route for high protein production by using less solution and alkaline chemicals (Espinosa-Ramírez & Serna-Saldívar, 2019). However, dry fractionation has been demonstrated as a viable industry approach for navy bean, fava bean, lentil, smooth pea, wrinkled pea, mung bean, and lima bean (Cloutt et al., 1986; Ningsanond & Ooraikul, 1989; Tyler et al., 1984), and chickpea (Xing et al., 2020). The dry fractionation approach is coupled with air classification and is used to produce starch and protein-enriched products. For example, according to Xing et al., (2020) air classification increased protein content in fractions by about 59% compared to whole chickpeas. The separation was impacted by the aerodynamic properties of particles, i.e., density and particle size (set point of 20 µm). In contrast, the milling parameter dictates the protein and starch characteristics and yield of the fractions. However, the final protein concentration obtained requires enough small particles and a higher milling speed to separate protein properly from starch. The disadvantages of this method are excessive fractionation to attain small particles leads to damaged starch that negatively impacts flow and poor behavior of flow and separation from protein. In addition, higher lipid-containing pulses such as chickpeas reduce particle dispersibility (Fernando, 2021).

Electrical forces have been applied in the electrostatic separation method which separates particles based on their charge. Electrostatic separation is recently used in starch-containing legumes such as chickpeas. Results indicated no protein enrichment was observed for chickpeas (Xing et al., 2020).

1.5.2. Wet fractionation

Wet fractionation is the most effective technique and can increase protein and starch purity. Unlike dry fractionation, wet fractionation involves soaking, washing, and drying steps. Although this method results in fractions high in protein or starch, a significant water resource is needed. Commercial wet milling commonly employs water or sodium hydroxide (NaOH) for isolating protein. In contrast, laboratory wet milling includes soaking steps to isolate starch that include a series of the solutions, such as NaOH, sodium bisulfite, sodium metabisulfite, and Tris-HCl buffer, while water, ethanol, and acetone are used as wash-out steps to remove impurities were applied for wash out protein (Zhao et al., 2020). An example of chickpea flour involves soaking in 0.45% sodium bisulfite for 6 h and at room temperature. Then, followed by centrifugation at 4000 g for 10 min. The sediment is collected, and these steps are repeated for an ethanol wash and dried in the oven (Tan et al., 2022). With pH, the increase can dissolve protein content while starch can be collected after every centrifuge step separating non-white layers. In contrast to starch isolation, protein isolation, and purification can be accomplished using several approaches as defined below. The most effective method to isolate protein is alkaline extraction which is used by industrial protein isolation. There are many disadvantages for alkaline extraction such as water waste that has sustainability concerns. Nowadays, salt extraction has been taken advantage of compared to alkaline extraction due to its environmentally clean procedures.

1.5.2.1. Alkaline extraction

The process of the alkaline extraction of protein from chickpeas has been commonly performed at pH 10.5 and 50 °C with a 1:10 (w/v) ratio of solid: liquid for 1-2

h. However, an adaptation of this approach is common. One such adaptation includes the use of enzymes (0.06 U arabinofuranosidase/g DM or 34 U xylanase and 17 FPU cellulase /g DM) during the alkaline extraction. This approach resulted in the highest protein recovery (~ 21%) and about 93% extraction efficiency (Perović et al., 2022). There are many advantages and disadvantages in alkaline extraction, for example, protein denaturation and Maillard reaction that results in brown substances. Also, intermolecular cross-coupling at a high concentration of alkaline solution leads to protein rearrangement. However, an increase in the total extraction yield at high pH justifies this extraction method. The high pH neutralizes side amine groups of lysine, arginine, and other basic amino acids and facilitates better extraction of the protein (Gao et al., 2020).

The isoelectric point (pI) of the chickpea protein is used as an approach to precipitate alkaline extracted proteins. In this approach, adjustment of pH to 4.3 (pI of chickpea protein) results in a precipitated protein that has a purity usually greater than 80%. However, the loss of soluble proteins generally results, and thus overall protein recovery is often lower than expected. The protein content of laboratory-prepared protein isolates is about 89.9% to 94.4% (Table 1.4) from different chickpea cultivars (Kaur & Singh, 2007). The isoelectric precipitation approach at the laboratory scale results in protein isolates with 85.4% (Karaca et al., 2011) to nearly 90% (Glusac et al., 2020) purity.

Table 1.4. Protein content (%) and protein isolates (%) in different cultivars of chickpea (Kaur & Singh, 2007).

Cultivars		Protein (%)	Protein isolates (%)
	PBG-1	23.7^{b}	94.3^{a}
	PDG-4	20.6^{a}	92.8^{ab}
Desi	PDG-3	23.9^{b}	93.3^{ab}
	GL-769	24.3^{bc}	89.9^{a}
	GPF-2	22.3^{ab}	91.6^{ab}
Kabuli	L-550	26.7^{c}	94.4^{b}

Lowercase letter indicates significant ($p \leq 0.05$) different Whitin the columns.

The general approach to protein isolation is to extract (90 min at 500 rpm) the protein flour source with NaOH (1 M) solution at pH 9.0 in a ratio of 1:10 (w/v flour to NaOH solution). After centrifugation at 4500 x g for 20 min, the supernatant is retained for subsequent isoelectric precipitation. The pellet can be reextracted as described previously to enhance protein recovery. The isoelectric precipitation of the combined supernatants is done by adjusting pH to 4.3-4.6 and protein is recovered by centrifugation 4500 x g. Subsequent adjustment to pH 7.0 results in a soluble protein solution that is then dried. In laboratory extractions, freeze-drying is commonly practiced while spray-drying is a common industrial practice. Another isolation of protein is based on extraction with sodium sulfite at pH 10.5 and washing with distilled water to reach pH 4.3, followed by ethanol and acetone washing (Boye et al., 2010). Although this method is suitable for laboratory preparations, it is not well suited for commercial protein isolation.

1.5.2.2. Salt extraction

Chickpea protein has a high number of salt-soluble proteins including albumin and globulin. Particularly, salt extraction can isolate higher albumin concentration in the final solution compared to alkaline extraction, and salt extraction results in higher protein purity, lighter color products, and better emulsification properties (Hadnađev et al., 2018). Optimal salt extraction protocols were utilized for defatted flour. The defatted flour was

mixed with a salt solution such as a 5% potassium sulfate aqueous solution (1:10 ratio w/v) (Glusac et al., 2020) or sodium phosphate (0.1 M) (Karaca et al., 2011) at a pH of 7.00 using NaOH (0.1 M) and held for 1 h under constant stirring at 500 rpm. The supernatant was collected after centrifugation at 17,700 x g for 20 min and dialyzed for 72 h using Milli-QTM water. After dialysis, freeze-drying is commonly performed. Salt-extraction method had a greater protein recovery yield (44.5%) and a higher extraction yield (32.5%) compared to alkaline extraction in hemp protein isolation (Fang et al., 2023).

1.5.2.3. Organic solvents extraction

Another laboratory protein extraction applied to chickpeas is based on organic and aqueous solvents using methanol, ethanol, ammonium sulfate, acetone, citric acid, hydrochloride acid, and trichloroacetic acid (Kumar et al., 2021). Extraction of proteins that have lipid binding and nonpolar chains using organic solvents (acetone, butanol, and ethanol) indicates an advantage over alkali solutions due to the lipophilicity and hydrophilicity ability of organic solvents at the same time. The main problem of protein extraction from plants is cell walls that are not water-soluble, and have hydrophobic groups. However, the isolation using alkaline solution, such as 0.1 mol/L NaOH, is better at degrading cell walls and thus is favored for isolating protein over organic solvents (Cui et al., 2017). For organic extraction of protein, alcohols, buffers, or strong denaturants are used. Many solvents are specific for different products and different purposes resulting in pure protein products. However, protein recovery is generally low.

1.5.2.4. Ultrafiltration

Ultrafiltration is mainly separation based on size. The hydrodynamic radius of proteins can be controlled by the selection of the buffer. Ultrafiltration could be a

replacement for isoelectric precipitation since the proteins still need to be extracted from the flour source, and non-soluble material filtered before the ultrafiltration step. For example, protein extraction can be done using 0.05 M sodium hydroxide in the ratio of 1 to 20 (w/w) dry basis. After heating (60 °C) and stirring, the mixture is centrifuged at 400 x g. The supernatant is collected and filtered to avoid having non-soluble contamination and then, it is ultrafiltered by using a polymeric membrane under pressure (e.g., 1.73 bar) and the resulting concentrated supernatant is spray or freeze-dried (Alfaro-Diaz et al., 2021). Heat treatment may change the protein structure and lead to a loss of nutritional and functional properties. Diafiltration is applied to the production of whey protein concentrate in small volumes several times (Baldasso et al., 2011).

Ultrafiltration has been used to isolate the protein of peas, chickpeas, and lentils to evaluate functional properties (Boye et al., 2010). Extractions were applied to adjust pH at 9.5 with a 1:5 solid/liquid ratio at 35 °C for yellow peas and chickpeas and pH 9 and a 1:10 ratio of solid/liquid at 25 °C for lentils. Supernatants were passed through a 50 kDa MWCO with diafiltration (4X) at pH 6 (Boye et al., 2010). Mondor et al. (2009) reported the concentration of the pH 9.5 extracted chickpea (1:9 ratio flour to liquid) solution. After centrifugation at 1700 rpm to remove insoluble material. The solution was filtered with 50 kDa MWCO, centrifuged at 1000 × g, and freeze-dried. The process of defatting did not affect antinutritional factors while isoelectric precipitation increased phosphorous and phenolic contents. Ultrafiltration in isoelectric protein isolation results in lower trypsin inhibitor.

1.6. Protein and starch extraction on an industry scale

Plant-based protein market grow globally by 7.3% from 2022 to 2027 and estimated to reach USD 12.2 in 2022 and will reach USD 17.4 billion by 2027 (Anonymous, 2022). According to GEA company, extraction, separation, purification, concentration, and drying technologies exploit protein concentrates to isolates. For example, in wet separation methods, decanter centrifuge and membrane filtration are used for high recovery levels of protein. Also, in some cases protein-rich fractions derived from the isolation and purification process are neutralized and dried using a spray dryer (GEA, 2023).

1.7. Analytical methods

1.7.1. Electrophoresis of protein fractions

Polyacrylamide gel electrophoresis is one of the important methodologies used to examine protein fractions. Electrophoresis is applied using 4% acrylamide stacking gel and 10% separation gel. Samples are added after the dried protein extract is reconstituted in a buffer consisting of 1.5 M Tri-HCl, glycerol, and 1% bromophenol blue at pH 8.8. The examination is comprised of standard protein markers that include, 669,000 Da thyroglobulin, 440,000 Da ferritin, 232,000 Da catalase, 140,000 lactate dihydroxygenase, and 66,000 Da albumin. The gel also is fixed with a fixing solution of water, methanol, and acetic acid (7.2.1 ratio). After 3.5 hours of protein migration, the gel is kept for 30 min for fixing and 1 h in Coomassie Brilliant Blue R250 (Davis, 1964).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis utilizes 12% resolution gel and 8% stacking gel, 0.5 M Tri-HCl pH 6.8, 10% v/v glycerol, 2% w/v SDS, 0.01% w/v bromophenol blue and 3% v/v β-mercaptoethanol. Before applying the samples in wells, prepared solutions are heated at 98 ℃ for 10 min and centrifuged at 13,600 g for 10 min. Also, a standard molecular ladder contains a molecular weight from 10 to 245 kDa. The process of migration takes 1.5-2 h at 35 mA. Staining and de-staining are the same as native PAGE (Laemmli, 1970). For de-staining, 20% methanol and 10% acetic acid solution for 3 h is used. chickpea protein isolation yielded 8 major proteins composed of 71, 44, 37, 28, 24, 22, 16, and 12 kDa (Sofi et al., 2020). The 70, 50, 35, and 22 bands could be considered as convicilin, α, β, γ-vicilin, basic β, and 11S proteins, respectively.

One of the important differences between non-reductive and reductive SDS-PAGE involves applying β-mercaptoethanol, which promotes the reduction of disulfide bonds, causing crosslinked proteins to break. In electrophoresis, this results in the disappearance of larger proteins. Disulfide crosslinked proteins (basic β) are responsible for aggregates of large proteins that occur during the pH shift during isoelectric precipitation (Wang & Xu, 2022). In reductive SDS PAGE due to the presence of the mercaptoethanol disulfide bonds between cysteine residues in 11S and 7S globulin fractions are reduced, which also destroys the quaternary structure of the protein having disulfide bonds. So, in the reductive method, more bands can be detected (Papalamprou et al., 2009; Wang et al., 2020). According to Li et al. (2021) bands of 15, 18, 34, and 70 kDa are attributed to the vicilin subunit of 7S protein, and 10, 12, and 41 kDa are attributed to the legumin subunit 11S protein.

1.7.2. Electrophoresis of protein

The capillary gel electrophoresis technique arose from the chromatography technique focusing on isoelectric and size separation. Starch gel separation is the primary gel separation followed by SDS polyacrylamide gel electrophoresis. Separation of the amines, amino acids, and dipeptides has been achieved by using a 75 µm internal diameter tubular capillary and a column fluorescence detector with up to 30 kV. In addition, the use of smaller capillaries affects the surface area-to-volume ratio and efficient heat dissipation. Proteins migrate under the electric field application into capillaries which are connected to the electric reservoir on both ends leading to separation based on size. Gels always are made from polymers, buffers, detergents, reducing agents, and organic compounds (Bhimwal et al., 2022; Zhu et al., 2012). Freeze-dried protein isolation of gluten-free flour such as buckwheat, white rice, and teff batters was analyzed by using lab-on-a-chip capillary gel electrophoresis to observe cross-linking mechanism and protein polymerization under high-pressure treatment (Vallons et al., 2011). Protein extraction from gluten-free cereals of brown rice, maize, and teff was characterized by capillary gel electrophoresis using different buffers. Capillary gel electrophoresis indicated advantages over SDS-PAGE due to the high resolution of peaks and quantifying ability using an internal standard (Moroni et al., 2010).

1.7.3. FTIR of protein

Spectra of the isolated protein of Fourier Transform Infrared spectroscopy (mid-IR) in the range of 4000-800 cm^{-1} is used for calculating the percentage of α-helix (1650-1660 cm^{-1}), β-sheet (1630-1638 cm^{-1}; 1625 cm^{-1}; 1679 cm^{-1} and 1695 cm^{-1}), β-turn

(1669 cm^{-1}) and disordered structures (1639 cm^{-1}) (Espinosa-Ramírez & Serna-Saldívar, 2019). Chickpea comprises 19.9% α-helix, 37.2% β-sheet, 7.7-18.5% β-turn, and 7.2% antiparallel β-sheet (Carbonaro et al., 2012). For example, the reduction in β-sheet and α-helix structure in extruded isolated pea protein compared to raw material was evaluated using FT-IR. Results supported that a portion of the β-turn was increased from 9.7 ± 0.01% up to 13.4 ± 0.13% at the 400 rpm screw speed of the extruder and the β-sheet indicated an increase. Changes in β -structures indicate an increase in the digestibility of pea protein (Beck et al., 2017). According to (Onder et al., 2022), β-sheet is a predominant structure for Kabuli-type chickpeas, and amide I (band 1610-1700 cm^{-1}) was observed as a secondary structural component.

1.7.4. FTIR of starch

Fourier transforms infrared (FT-IR) spectroscopy is applied by blending samples with potassium bromide powder and pressed into tablets. Also, calibration is set using potassium bromide with a recording range of 600-4000 cm^{-1}. The C-O-H band at 1000-1022 cm^{-1} correlates to the amorphous state in starch which is the intermolecular hydrogen bonding of hydroxyl groups. Extreme broadband at 3235-3290 and 2900-2925, 1636, 1336, and 1144 cm^{-1} is attributed to O-H stretching vibration, C-H band, H-O-H, O-C-H and C-C-H, and C-O and C-C bands respectively. However, different ratios of amylose to amylopectin result in different peak intensities in the spectra (Bitik et al., 2019).

1.8. Starch characteristics

1.8.1. Granule morphology

The granular structure of the different chickpea cultivars and varieties indicates significant differences in shape and size (varying from 7 to 29 µm). The average length and width of granules ranged from 22.0-22.4 µm and 18.5-18.8 for Kabuli type and 17-20.1 and 11-14.4 µm for Desi types, respectively (Hoover & Ratnayake, 2002; Miao et al., 2009). Granules appear smooth with the shape of the cobble and spherical due to the differences in the biological origin of the amyloplast. Concentric layers were observed, which exhibit higher intermolecular organization and high content of the crystalline zones (Miao et al., 2009). Also, under polarized light, a well-defined birefringence pattern (which resulted from bent polarized light due to high molecular order) was observed and led to the conclusion that a high degree of molecular orientation in granules existed (Miao et al., 2009). Starch granule proteins 1, -2, and -3 are related to 100-105, 90, and 77 kDa granule-bound isoforms, respectively. Furthermore, 100-105 kDa proteins bound to starch granules are detected at an early stage of endosperm development (Van Hung et al., 2006). Legume starches show a single restricted swelling and low amylose leaching. This might be due to the strong interaction between starch chains and high amylose content, which are closely packed. Within the granule, Legume starch gelatinizes in excess water and high temperature and after cooling, starch chains reorient to form an ordered structure during retrogradation. Retrogradation causes an increase in crystallinity degree, firmness of gel, and syneresis (Hoover et al., 2010).

1.8.2. X-ray diffraction of starch

The XRD test is run using an analytical diffractometer with a pixel detector having CuKα and running at 54 kV and 40 mA (Sun et al., 2014). The sample analysis range is 2θ = 2 to 40° with a step interval of 0.05 and a scan rate of 2°/min. The intensity of peaks in a diffraction pattern is associated with the presence of ordered crystalline structures and the electron density distribution between the amorphous and crystalline regions. Sharp peaks indicate the presence of well-defined crystalline regions, whereas diffused peaks represent the presence of the amorphous region. In general, different crystallinity of starches is related to crystalline size, a portion of the crystalline region, double helices orientation, and double helices interactions. The semi-crystalline starch granules have an impact on gelatinization and glycemic responses (Sun et al., 2014).

The X-ray diffraction test showed strong diffraction peaks at 15°, 17°, 18°, and 23°, for 2θ from different cultivars of Kabuli and Desi types (Hoover & Ratnayake, 2002). The peaks from the diffractogram indicated that chickpea starch granule is C-type (has a resemblance to type A and B at the same time) which is the mixture of the A and B units, and the peak pattern depends on the starch origin and environmental growth condition. Kabuli starch had a higher degree of crystallinity compared to the Desi type, likely due to the different content of the amylopectin and the increase of the amylose leading to a decrease in the double helix content (Hoover & Ratnayake, 2002; Polesi & Sarmento, 2011). A negative relationship was detected between crystallinity and amylose concentration (Sandhu & Lim, 2008). For example, germination led to a decrease in relative crystallinity (4-6% decrease) due to the re-organization of starch pattern structure and hydrolyzation of granules by amylases (Sofi et al., 2023)

1.8.3. Differential scanning calorimetry of starch

Differential scanning calorimetry (DSC) shows the thermal behavior of the starch-binding water system. The important points are onset temperature ($T_{o\text{-}D}$), peak temperature ($T_{p\text{-}D}$), and heat of gelatinization (ΔH) were established and the degree of gelatinization by DSC (DG_D) can be calculated. The general approach includes the addition of 2 mg of starch flour placed in a stainless-steel pan with the addition of deionized water. Then, pans are heated from 25 °C to 120 °C at a rate of 5 °C/min with the use of indium and empty aluminum pan calibration. The thermograms indicated two peaks. Peak 1 is associated with starch gelatinization, while the second peak is attributed to protein denaturation and amylose-lipid complexation (Chigwedere et al., 2018; Noordraven et al., 2021). Additional DSC data of chickpea starch for T_o, T_p, T_c, and ΔH were reported 63-68 °C, 69-73 °C, 76-80 °C, and 3-8 J/g, respectively (Bashir & Aggarwal, 2017) indicated that Kabuli-type starch had the lowest thermal values compared to Desi chickpeas starch. In a study of different seed starches, such as adzuki bean, chickpea, faba bean, and baiyue bean grown in China, the lowest T_o, T_p, and T_c values were observed for chickpea and suggest a lower degree of crystallite stability (Zhang et al., 2019b). Lower transition temperature and gelatinization enthalpy for chickpeas were theorized to be due to the small content of long-chain amylopectin and the high content of short-chain amylopectin molecules (Zhang et al., 2019b).

1.9. Functional properties of chickpea starch and protein

1.9.1. Pasting properties

The pasting properties of starches are commonly observed using a rapid viscosity analyzer (RVA). The specific amount of the flour is mixed with water depending on the sample moisture content and the instrument starts to heat the mixture, holding the temperature at 95 ℃ for 5 min, and cooling to 50 ℃ with a constant stirrer at 160 rpm. The outcome curves give peak viscosity (mPa.s) which measures crystallinity, trough viscosity (mPa.s), final viscosity (mPa.s), setback viscosity (mPa.s), and pasting temperature. These values for native chickpea starch were 1201.0 ± 23.52 mPa.s, 1005.0 ± 18.02 mPa.s, 1565.0 ± 14.29 mPa.s, 551.6 ± 10.40 mPa.s, 76.51 ± 0.50 ℃, respectively (Bashir & Aggarwal, 2017). The pasting temperature ranged between 73.05-75.20 ℃ indicating lower resistance to swelling and rupturing while peak viscosity, final and breakdown viscosity were 1348-2163 mPa.s, 1515-2704 mPa.s, and 71-269 mPa.s, respectively (M. Kaur & Singh, 2007). According to Tan et al. (2022) achievements, the pasting properties of protein fractions and starch of the chickpea are different. For example, starch, starch with total purified protein, starch with globulin, and starch with glutelin had different peak viscosity at 623.6, 937.5, 849.5, and 1119.0 (mPa.s), respectively. Breakdown viscosity indicates the thermal stability of gel after reaching 95 ℃ that means having lower breakdown viscosity shows higher stability to phase separation (Tan et al. 2022). The results suggested that globulin with the starch sample is very stable under heat and glutelin with the starch sample susceptible to phase separation.

1.9.2. Emulsion and foaming properties

Emulsion capacity refers to the amount of oil that can be emulsified by protein (mainly albumins) in water and emulsion activity is the ability of protein to form an emulsion (Karaca et al., 2011). Chickpea flour was introduced as a stabilizer studied as a non-crosslinked emulsion which was stable over 2 weeks of storage (Glusac et al., 2020). Foam formation involves two phases: a dispersed phase and a continuous phase. Polysaccharides, owing to their hydrophilic properties, remain in the aqueous phase. The foaming properties are significantly influenced by the concentration of flour, which in turn increases protein concentration and promotes protein-protein interactions. These interactions play a crucial role in bubble formation and the creation of multilayer protein films, which contribute to foam stability. For achieving highly stable foam, water-soluble and native protein extraction methods should be employed from chickpea flour to minimize the negative impacts of other components such as lipids (Kaur & Singh, 2005). This approach ensures the retention of proteins in their natural state, enhancing their ability to contribute to foam stability over time (0-120 min).

1.9.3. Water holding and oil holding capacity

Water holding capacity is the amount of water absorbed per gram of protein/starch or flour. Protein isolates are important in bakery, meat, and soup products due to their importance in keeping water into molecular structure and texture (Onder et al., 2022). In addition, OHC is the amount of oil that can be absorbed by nonpolar amino acids and thus is an important function if protein in meat systems. According to Onder et al. (2022), the WHC of chickpeas ranged from 2.1 to 2.7 g/g and the isolates with higher carbohydrate/uncharged polar amino acids had lower WHC compared to the isolates with

lower carbohydrates. Also, a positive correlation was reported between carbohydrate/uncharged polar amino acids with OHC which was reported between 1.3 and 4.1 g/g. The addition of globulin and glutelin to chickpea starch leads to a significant increase in peak viscosity, setback viscosity, and final viscosity indicating that protein-starch mixture results in higher WHC and forms firmer gel paste (Tan et al., 2022). M. Kaur & Singh (2005) observed presence of hydrophobic proteins tends to bind with lipids and have high Oil holding capacity (OHC) is driven by a non-polar amino acids side chain that binds to the paraffin chain of fatty acids.

1.9.4. Gelling capacity of the protein

Gelling capacity is the method to determine the gelling potential of the protein by using the least gelling concentration (LGC). Gelling capacity is important in food applications because a semi-solid structure is used in plant-based products such as plant-based meat, sausage, and meat products with pH 7, and different concentrations are provided from 10 to 20% (W/V) (Ma et al., 2022). Solutions are heated for 1 h at 90 °C and cooled at 4 °C immediately. The least gelling concentration is determined with minimum protein concentration which does not show semi-solid gel characteristics and the firm gel has self-support and no flow on inversion. Texture profile analyses are used to measure the hardness, adhesiveness, springiness, cohesiveness, and gumminess of gels. Gelling capacity depends on pH, ionic strength, heating, and protein sources. In this regard, chickpea protein has lower LGC (5-7%) compared to other pulse proteins.

1.10. Application

Several food applications comprise starch, protein, flour, and other fractions of chickpeas (Table 1.5). These applications show the effects of the contribution of the chickpea fractions in different products. Chickpea flour and protein incorporation in food products leads to increased nutritional values and affected textural properties while functional properties improved due to having higher protein content (Gómez et al., 2008; Thushan Sanjeewa et al., 2010).

Table 1.5. Important application of chickpea protein, starch, and flour.

Product	Results	Description
Chickpea bread (Mohammed et al., 2014)	Dough development and dough stability were significantly higher ($P < 0.05$) and dough strength is weak for chickpea flour.	Combination of different proportions of wheat and chickpea flour.
Chickpea bread (Miñarro et al., 2012)	Chickpea flour showed the highest volume increase and lowest hardness of loaf compared to other protein sources.	Different proteins sourced such as chickpea flour, pea protein, carob germ flour, and soya flour were replaced.
Chickpea biscuits (Yadav et al., 2012)	Biscuits with a high ratio of chickpea flour indicated the highest protein, fat, ash, protein contents, Oil holding capacity, foaming capacity, and water absorption capacity.	Biscuits were developed by adding different ratios of chickpea flour.
Chickpea biscuits (Lu et al., 2022)	Compare the chickpea starch digestibility in developed biscuits.	Functionality properties such as water solubility and water absorption increased, and swelling power were decreased in chickpea flour biscuits.
Chickpea biscuits (Rababah et al., 2006)	The addition of chickpea spread ratio and liking attributes decreased, and yellowness (b*) increased.	The effects of chickpeas with different concentrations were evaluated on the physicochemical and sensory properties of biscuits.

Product	Results	Description
Chickpea biscuits (Mieszkowska & Marzec, 2016)	Chickpea flour increases yellowness and hardness. 20% of chickpeas and 40% and 60% of polydextrose received the highest scores.	Evaluate the replacement of sucrose by polydextrose and inulin in chickpea biscuits.
Chickpea biscuits (Schouten et al., 2023)	20-40% of chickpea flour decreased the acrylamide formation despite having a high amount of asparagine.	Chickpea flours were replaced in different ratios to observe the formation of acrylamide due to the thermal stability of chickpea protein.
Chickpea pancake (Necheporuk et al., 2021)	Daily requirements of phosphorus, potassium, iron, and calcium were covered by using chickpea flour.	In different treatments, chickpea flour is replaced completely with wheat flour.
Chickpea products (Yust et al., 2010)	Solubility, oil absorption capacity, and foaming capacity of chickpea protein increased with hydrolyzing 5% peptide bonds.	Observation on effects of alcalase-glyoxyl derivatives on chickpea protein.
Chickpea pasta (Saget et al., 2020)	The incorporation of chickpea flour in pasta production leads to environmental sustainability and an increase in nutritional quality.	Chickpea flour in pasta formulation.
Chickpea cake (Gómez et al., 2008)	The lower pasting values for chickpea compared to wheat flour were observed due to the lower carbohydrate content of the chickpea. Chickpea flour increased the batter density of cakes due to less air incorporation. The fiber and protein content of chickpea flour cake was significantly increased.	Effects of chickpea flour replacement on the Cake.
Aquafaba (Mustafa et al., 2018)	The foam of egg white and aquafaba is comparable regarding foam volume and foaming capacity. Aquafaba cake had a lower height and volume index due to having heat-stable proteins that cause a different mouth feel.	Observation on egg white and aquafaba properties and their application on the cake.

Product	Results	Description
Aquafaba **(He et al., 2019)**	Aquafaba emulsion capacity and stability ranged from 1.10 to 1.30 m^2/g and 71.9 to 77.1% respectively in different cultivars.	Production of aquafaba using different cultivars.
Aquafaba **(Meurer et al., 2020)**	Foam expansion was 84% higher value for 100% ultrasonic equipment power compared to not treated aquafaba due to the decrease in particle size of protein aggregates. Foam stability also increased with ultrasonic treatment.	Application of ultrasound to extract aquafaba with significant functional properties.
Aquafaba **(Lafarga et al., 2019)**	Water content and pH had significant effects on foaming and emulsifying properties. Hydrophilic-lipophilic balance depends on lower pH which affects aquafaba properties. The optimum pH was obtained at 3.50.	Aquafaba properties were controlled with boiling conditions and pH.
Aquafaba **(Raikos et al., 2020)**	According to microstructural analysis, droplets of oil were densely packed and indicated a certain degree of polydispersity and stable mayonnaise structure.	Evaluate aquafaba on textural, composition, and stability of mayonnaise.
Chickpea flour **(Thushan Sanjeewa et al., 2010)**	Kabuli and Desi varieties of chickpea extended physiochemical, textural, and sensory properties.	Effects of the chickpea flour in low-fat pork bologna.
Chickpea splits **(Pathania et al., 2017)**	Chickpea was replaced at a ratio of 25, 50%, 75%, and 100%. Higher nutritional values and antioxidants were evaluated from snacks.	Formulation of flatbreads.
Chickpea flour **(Wang et al., 2023)**	High nutritional value and antioxidant contribution were recorded in the treatment of 46.5% chickpea and 55.6% oat flour.	Effects of chickpea flour on a protein-enriched biscuit.

Product	Results	Description
Chickpea starch (Zhang et al., 2019b)	Verities had c-type x-ray patterns but different swelling power, solubility, light transmission, pasting properties, and thermal parameters.	Compositional, morphological, and physicochemical properties.
Chickpea starch (Bashir & Aggarwal, 2017)	Irradiation caused a decrease in pasting properties and functional properties increase.	Effects of gamma irradiation on functional properties of starch.
Chickpea starch (Singh et al., 2004)	Starches from different varieties had granule length and width of 17-20 and 11-14 μm, respectively.	Evaluation of separated starch characteristics from different cultivars.
Chickpea protein (Chang et al., 2022)	The vicilin fraction had higher solubility, foaming, and emulsification properties.	Characterization, functional, and isolation properties were evaluated.
Chickpea protein (Karaca et al., 2011)	Chickpea protein had a negative charge at neutral pH.	Emulsifying properties of chickpea protein extracted from isoelectric precipitation and salt extraction.
Chickpea protein (Mondor et al., 2009)	Antinutritional factors such as trypsin inhibitors did not change in isoelectric precipitation while ultrafiltration decreased trypsin inhibitors.	Isoelectric precipitation and ultrafiltration effects on chickpea protein.
Chickpea protein (Sofi et al., 2020)	Enriched dough showed pseudoplastic behavior and glycemic index and digestibility decreased.	Enrich noodles with chickpea protein.
Chickpea protein (Tavano & Neves, 2008)	Native vicilin-like globulin was hydrolyzed partially with enzymes.	Isolation and hydrolysis of chickpea vicilin-like protein.
Chickpea protein (Yust et al., 2010)	The solubility of hydrolyzed proteins increased.	Functional and improvement of chickpea hydrolyzed protein with alcalase.
Chickpea protein (Kou et al., 2013)	Chickpea peptides had higher antioxidant activity.	Antioxidant peptides purification from albumin hydrolysates.

Product	Results	Description
Chickpea protein (Papalamprou et al., 2009)	Different methods affected functional properties, fractionation, and gelling behavior.	Effects of Preparation on isolated protein properties.
Chickpea protein (Wang et al., 2020)	Isolated proteins had higher solubility, emulsifying, and water holding capacity.	Effects of high-intensity ultrasound on protein isolate.
Chickpea protein (Wang et al., 2022)	Extreme decrease or increase of pH raised foaming ability.	Effects of ultrasound and pH shifting on protein properties.
Chickpea protein (Wang et al., 2023)	Chickpea protein inhibits myofibrillar protein degradation and oxidation.	Effects of chickpea protein as a cryoprotectant in frozen surimi.
Chickpea protein (Wang et al., 2023)	Emulsion stability improved and hardness increased.	Effects of chitosan and protein combination on phosphate-free pork meat emulsion.
Chickpea protein (Grasso et al., 2022)	Chickpea-based samples had higher adhesiveness, springiness, and cohesiveness.	Formulation of cheese using Chickpea protein.
Chickpea protein (Wang et al., 2023)	The myosin gel with 6%-9% chickpea protein had higher viscoelasticity, gel strength, hardness, water holding capacity and whiteness.	Effects of Chickpea protein on hairtail fish myosin gel

1.11. Conclusions

Chickpea flour surpasses wheat flour in protein, fat, ash, and fiber content, making it a valuable source of essential micronutrients. Variations in protein and fat content are notable among different chickpea types, particularly Kabuli and Desi varieties. Chickpea protein is rich in essential amino acids and unsaturated fatty acids, with major protein fractions such as albumin, globulin, prolamin, and glutelin contributing significantly to its

nutritional profile. Despite slightly lower levels of sulfur-containing amino acids compared to wheat, chickpea protein exhibits superior essential amino acid profiles. Processing techniques like baking, cooking, germination, and fermentation can optimize protein quality and digestibility. Chickpea starch, composed of amylose and amylopectin, contains higher amylose percentage in Kabuli type than Desi. Resistant starch in chickpeas offers health benefits including prebiotic properties, aiding in weight management, glucose metabolism, and lipid regulation. Ingredient production from chickpea to isolate protein and starch involves dehulling, soaking, or fractionation with chemical extraction methods utilizing alkali or organic solvents. Optimizing protein extraction techniques requires exploring factors like pH, temperature, and solvent selection. Analytical methods such as electrophoresis, Fourier Transform Infrared spectroscopy (FT-IR), X-ray diffraction (XRD), and differential scanning calorimetry (DSC) aid in understanding protein and starch characteristics. Further research is needed to refine processing methods and fully utilize the potential of chickpeas in the food industry.

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Chapter two

Effects of storage regimes on chemistry and functionality properties of different varieties of chickpea

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2. Abstract

The impact of storage conditions, such as 40 °C, high humidities (40%, 55% and 65%), and storage (0, 180, and 360 days) on nutritional value, pasting, and functional properties, color differences, and protein and starch quality of five Chickpea (*Cicer arietinum*) varieties (Crown, Royal, Sierra, Orion, and Frontier) were determined. The Sierra cultivar had the highest initial moisture content (MC, 7.7 ± 0.01%) and MC increased over time for all samples stored at 55% and 65% RH and 20℃. Protein (PC), total starch (TSC), and fat (FC) contents changed in all varieties during storage, but differences were not significant ($P \leq 0.05$). Under the same storage (65% RH and 40 ℃ treatment), the Frontier variety had significantly ($P \leq 0.05$) higher PC at day zero (24.0 ± 0% dwb) and 360 (23.9 ± 0.2% dwb) compared to other varieties, while no significant ($P \leq 0.05$) differences existed between other varieties. A general upward trend in the pasting data was observed for all varieties of the 360-day stored samples. In contrast, the gel firmness of the gels formed during RVA was lower for the 360-day samples. Emulsion capacity (EC) and Foaming capacity (FC) changed significantly ($P \leq 0.05$) in all samples over time under the effects of different variables. Color analysis revealed reduced yellowness in all samples during storage. Also, lightness values decreased over time, indicating seed darkening during storage. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed the disappearance of major protein bands around 37 and 55 kDa after 360 days, indicating protein aggregation and structural alterations. Fourier transform infrared spectroscopy (FTIR) of amid II structures indicated interactions and differences in the secondary structure of a protein in the samples stored for 360 days. Starch

analysis via SEM revealed protein-coated starch granules, indicating protein-starch interactions occurred during storage.

Keywords: Chickpea (*Cicer arietinum),* storage, harsh condition, chickpea protein, chickpea starch

2.1. Introduction

Chickpea (*Cicer arietinum*) is the third most important legume globally, following dry beans and dry peas (U. Singh et al., 1991). They have a high nutritional profile and fewer major anti-nutritional factors than other pulses (Chavan et al., 2009). Chickpeas are rich in protein (24.4%), dietary fiber (9.0%), complex carbohydrates (60.0%), folate, and trace minerals like iron, molybdenum, and manganese (Yeken et al., 2023). Chickpea protein has satisfactory water and oil holding capacity, emulsion and foaming capacity, and pasting properties. Moreover, chickpeas have been found to lower cholesterol and blood glucose levels (Pittaway et al., 2008). As a result, chickpeas can be incorporated into healthy diets to enhance overall well-being and reduce the risk of cardiovascular diseases and diabetes (Rehm et al., 2023).

Chickpea production was 15 Metric tons in 2021 with an average yield of 1016 kg ha^{-1} according to the Food and Agriculture Organization (FAO) of the United Nations (FAOSTAT, 2023), with the major chickpea production from India, Australia, Ethiopia, Turkey, and Myanmar. With the increasing importance of exports, maintaining seed, especially during post-harvest and storage. Integrity is essential for retaining the nutrition and functionality of the pulses. Storage conditions are crucial for extending shelf life and preserving quality (Rani et al., 2013).

Pulses can be harvested and stored in a wide range of temperatures (5-40 °C) and relative humidities (RH), where high temperature and RH lead to undesirable enzymatic activity and affect physical, chemical, and functional properties (Malhotra et al., 2023). Temperature, RH, moisture content (MC), and light were identified as the key factors affecting seed quality and color stability in legumes (Ellis et al., 1988; Wash Res et al.,

1975). Chickpeas are harvested when a MC drops to 18% but must be dried to <14% for safe storage (i.e., prevent molding, and browning). Preharvest, harvest, and post-harvest conditions play crucial roles in final nutritional, functional, and color properties. Relative humidity and temperature during storage must be controlled to maintain optimum quality. Elevated temperature accelerates deterioration, microbial growth, and nutrient breakdown (Chidananda et al., 2014). High humidity promotes mold and bacteria growth, causing spoilage. Combining high temperature and humidity worsens these effects. Therefore, storage of pulse ingredients in cool, dry conditions should be practiced preventing or mitigating negative effects associated with high temperatures and RH.

Chickpeas (*Cicer arietinum*) stored at 33-35 °C and 75% RH for 160 days had lower concentrations of phytic acid and seed coat tannin, in vitro digestibility, protein efficiency ratio (PER), net protein ratio (NPR), and water absorption index than control seeds (Reyes-Moreno et al., 2000). In contrast, an increase in cotyledon tannins, and darkening of the testa color, as exhibited by a decrease in Hunter 'L' value, and an increase in total color difference (ΔE) also was observed (Reyes-Moreno et al., 2000). Faba beans (*Vicia faba*) stored at high temperatures, moisture, and light intensity had darker colors and lower phenolic compounds, non-tannin phenolics, total tannins, and proanthocyanidins (Nasar-Abbas et al., 2009) due to polymerization of these compounds resulting in insoluble, and high molecular weight polymers. Also, some phenolic compounds might oxidize and produce dark degradations. Kabuli chickpea stored at several temperatures (10, 20, and 30 °C) and different RHs (55 to 95%) had no significant effects on protein content, however, a significant increase in free fatty acid content occurred (Malhotra et al., 2023).

Maize stored at 35℃ for 12 months compared to control had lower pasting temperature, setback, and final viscosity (Paraginski et al., 2014).

However, the impact of storage conditions on chickpea composition has not been widely published and may be responsible for composition differences observed in published literature. Furthermore, limited information on changes in protein structure and starch pasting properties has been reported. The objective of this study was to establish the impacts of storage on the composition and functionality of chickpeas after 360-day storage under various temperatures (21 and 40 ℃) and RH (40, 55, and 65%).

2.2. Material and Methods

2.2.1. Experimental design

The samples (Figure 1.1) were stored in sealed containers at room temperature (21-22℃) or 40℃ and varying RH levels of 40%, 55%, and 65% using humidity bags (Boveda® Two-Way Humidity Pack). The experimental design of this project was a factorial design with repeated measures (2×3). The humidity was checked every 2 days of storage using an RH meter (ThermoPro, USA) that had been placed in the storage containers at the time of seed and humidity bag placement into containers. If necessary, humidity bags were replaced if the RH percentage changed by more than 1% from the targeted RH. These conditions were chosen based on preliminary studies in the authors' laboratory and literature reports to ensure a safe environment for further experimentation without promoting mold growth.

Varieties, namely Orion, Frontier, Crown, Royal, and Sierra were stored in separate containers. At specific time points (days: 0, 180, and 360), samples were selected (120 g) from the containers (1 Gallon volume with 2000 g of sample) for composition and functionality analyses. To prepare the samples for detailed chemical and physical observation, the seeds underwent milling using a UDY Cyclone sample mill (Direct Drive 3010-014, Colorado, USA) with a milling screen size of 0.5 mm.

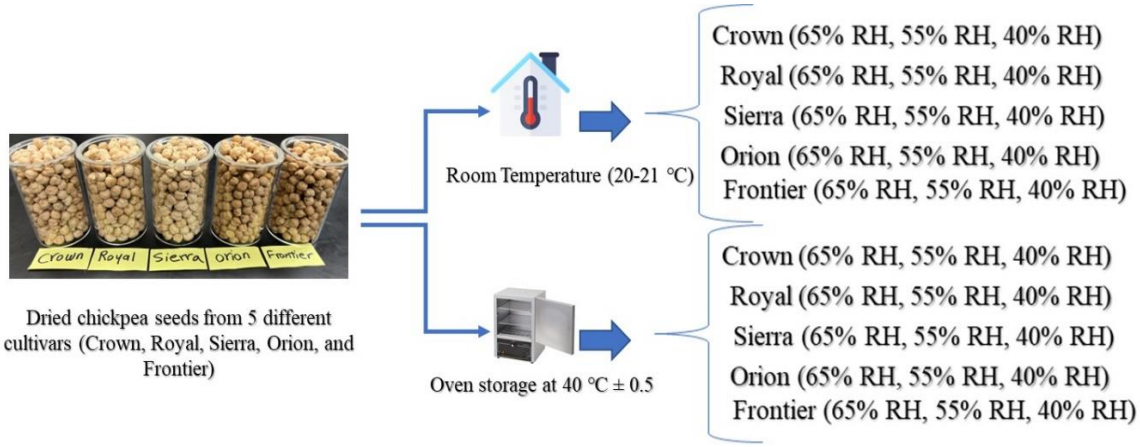

Figure 2.1. Storage conditions preparation of 6 different combinations of the storage condition of 2 different temperatures (21 and 40 °C) and different relative humidities (RH; 40%, 55%, and 65%) for 5 different varieties (Crown, Royal, Sierra, Orion, and Frontier). The high temperature of storage was prepared to keep samples in the oven and RH was arranged with moisture bags.

2.2.2. Chemical evaluations

Proximate chemicals (moisture, protein, fat, total starch, and ash) were measured using AACC Approved Methods of Analysis numbers 44-17.01, 46-30.01, 30-10.01, 76-13.01, and 08-01.01, respectively. The amylose content of starch was analyzed using a Megazyme kit (K-AMYL; Neogen, Lansing, MI). Non-starch carbohydrate was calculated by equation (1).

Non-starch carbohydrate (g/100 g) = (100 – moisture – ash – protein – starch – lipid) (1)

2.2.3. Protein and starch isolation

The isolated protein was prepared from chickpea seeds that were ground and subjected to isoelectric precipitation following a previously described procedure (Glusac et al., 2020). Chickpea flour was mixed with water (1:10 ratio) and pH was adjusted to 9.0 with NaOH. After stirring at room temperature, the mixture was centrifuged at 2500×g. The supernatant was collected, and the pellet was resuspended in water (1:5 ratio) with pH adjusted to 9.0 before the second centrifugation at 2500×g. The combined supernatants were acidified (pH 4) and centrifuged again to collect protein pellets that were then resuspended in pH 7.0 water. The samples were dialyzed in distilled water using a SnakeSkin^{tm} (3.5K MWCO, 22 mm I.D., Thermofisher, USA) filter tube and freeze-dried (Harvest Right, USA).

The remaining pellets after protein isolation contained starch that was used for starch isolation. The pellets were mixed with water (1:5 ratio), adjusted to pH 7.0, and homogenized at 8000 rpm (Fisher Scientific) for three cycles of 10 minutes each. The resulting solution was filtered through 60, 70, and 90 mesh sieves, and then centrifuged at 1700 × g for 15 minutes. After another sieving, the solutions were freeze-dried at -50 °C for 72 hours.

2.2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to determine the molecular weight (MW) of chickpea protein subunits, following the method described by Gao et al. (2020). The solutions, staining, de-staining, power supplies, electrophoresis chambers, ladders, buffers, and gels were from Bio-Rad (Hercules, CA).

The chickpea protein solution was prepared by mixing distilled water (2 mg/ml) and 2x Laemmli sample buffer with the addition of 2 µL Dithiothreitol. Before loading samples (15 µL) onto a 4-15% Mini-PROTEAN TGX precast gel, samples were heated at 95 °C for 5 min along with molecular weight standards ranging from 10 to 250 kDa (Bio-Rad). Electrophoresis was carried out at a constant voltage of 200 V for 30-40 min using a Bio-Rad Mini-Protein apparatus III until the dye front reached the reference line. Following electrophoresis, the gel was stained with a 0.05% (w/v) solution of Bio-Safe^{TM} Coomassie stain. Subsequently, the gel was de-stained with a Coomassie Brilliant Blue R-250 distaining solution. By comparing the protein bands with the molecular weight standards, the molecular weights of convicilin, vicilin, and legumin were determined.

2.2.5. Fourier transform infrared spectroscopy (FT-IR)

The FTIR (Nicolet 6700, Thermo Electron Corporation, WI, USA) spectra were acquired using a resolution of 4 cm^{-1} and 60 scans in the wavelength range of 400-4000 cm^{-1} and the background was collected before each sample scanning according to the previously described (Cui et al., 2020). Data analysis was carried out using Origin 2023

software (Originpro 2023b, OriginLab Corporation, MA, USA), including a subtraction process to remove water interference. Further calculations were performed to quantify protein secondary structure in the amide regions (1600-1700 nm). The resulting curve was smoothed and subjected to Gaussian deconvolution and areas of Gaussian peaks.

2.2.6. Surface hydrophobicity

The assessment of surface hydrophobicity was obtained according to the method Perovic et al. (2022) using bound bromophenol blue (BPB) that involved mixing 200 µL of BPB solution (1 mg/mL BPB) with a 2 mg/mL protein solution (1 mL). After 10 min holding time at room temperature, the mixture was centrifuged (2000×g) for 15 min. The absorbance of the resulting supernatants was measured at 595 nm against the phosphate buffer (20 mM). The BPB bound by protein was evaluated using Equation (2):

$$\text{BPBbound } (\frac{\mu g}{mg}) = 200 \frac{\frac{A_0 - A_1}{A_0}}{m_P} \quad (2)$$

where denotes the mass of protein measured (mg), 200 is the mass (µg) of BPB in analysis, A_o and A_1 represent the absorbance value observed for the control (200 µL BPB solution in 1 mL phosphate buffer) sample and the chickpea protein sample, respectively, at a wavelength of 595 nm.

2.2.7. Scanning electron microscopy (SEM)

The morphological characteristics of chickpea flour were obtained using a scanning electron microscope (SEM Hitachi S4700) with 2.5 nm resolution at 1.0 kV and 1.0k magnification range. The isolated starch samples were observed through drying and were

securely attached to aluminum stubs using double-sided sticky tape and the samples coated with a thin layer of gold.

2.2.8. Functional properties of flour

2.2.8.1. Pasting evaluation

The pasting properties of chickpea flour (CHF) were evaluated using the rapid visco analyzer (RVA, 4800, PerkinElmer, USA) following the modified method 61-02.01 of the AACC Approved Methods of Analysis (Perović et al., 2022). The modification included extending the protocol from 13 min to 23 min with the final 10 min being held at 50°C. The amount of flour and water (Chickpea flour (3.5 g) and distilled water (25 g)) were used according to the approved method. Peak viscosity (PV), hot paste or trough viscosity (TV), final viscosity (FV), setback viscosity (SB), breakdown viscosity (BD), and pasting temperature (PT) were recorded in centipoise (cP) units during the RVA test.

The gels obtained from the RVA run were cooled for 2 hrs at room temperature (21°C) and then analyzed using a texture analyzer (TA. XT Plus, Texture Technologies Corp., South Hamilton, MA, USA) equipped with a TA-10 probe to assess the gel firmness. Texture analysis was performed with a testing speed of 4.0 mm/sec, trigger force of 2.0 g, and 15.00 cm depth settings to determine gel firmness (g).

2.2.8.2. Water-holding and oil-holding capacity

Water holding capacity (WHC) was completed on chickpea flour (Nkurikiye et al., 2023). Chickpea flour (1.00 ± 0.01g) was placed into a centrifuge tube followed by 5 mL of water. After mixing with water, the flour was allowed to rest for 30 min. The mixture was centrifuged at 8000×g or 15 min. The water holding capacity was calculated according to equation (3):

$$WHC = \frac{(W_3 - W_2) + (W_1 - m_c)}{(1 - m_c) W_1} \quad (3)$$

where W_1 is the sample weight before water addition; W_2 is the weight of the syringe assembly plus hydrated sample; W_3 is the weight of the syringe assembly and sample after centrifugation; and mc is the initial moisture of the sample as a decimal (Nkurikiye et al., 2023).

Oil holding capacity (OHC) was evaluated with a method described previously (Sun et al., 2023), with slight modifications. Chickpea flour (2 g) and 1.5 mL oil were mixed in a test tube by vortexing twice (10 s every 10 min) for 30 min. The mixture was then placed in a syringe assembly with filter paper and centrifuged at 1000 x g for 15 minutes. The weight of the syringe assembly with filter paper and sample after centrifugation was recorded. The OHC was calculated using equation (4):

$$OHC = \left[\frac{W_3 - W_2 - W_4}{(1 - \frac{m_c}{100}) W_1} \right] \quad (4)$$

Where W_1 is the weight of the sample before oil addition (g); W_2 is the weight of syringe assembly (g); W_3 is the weight of the syringe assembly with added oil, W_4 is the weight of oil absorbed by the blank filter paper after centrifugation (g), and m_c is initial moisture (%) of the sample (Sun et al., 2023).

2.2.8.3. Water absorption index and water solubility index

The water absorption index (WAI) and Water solubility index (WSI) were completed following published methods (Singha et al., 2018). Briefly, chickpea flour (2.5 g) was mixed with 30 mL distilled water in a 50 ml centrifuge tube, vortexed for 30 seconds, and centrifuged at 1000 x g for 10 minutes. The supernatant was transferred to a

pre-weighed 150 mL beaker and dried in an oven at 135 ℃ for 12-16 hrs. The WAI and WSI were calculated by using equations (5) and (6):

$$WAI = \frac{Weight\ of\ the\ wet\ sediment\ (g)}{Initial\ weight\ of\ the\ dry\ flour\ (g)} \quad (5)$$

$$WSI = \frac{Weight\ of\ the\ solids\ in\ the\ dried\ supernatant (g)}{Initial\ weight\ of\ the\ dry\ flour\ (g)} \times 100 \quad (6)$$

2.2.8.4. Evaluation of foaming properties

Foaming capacity was determined following the method of Liu et al. (2010). A 1.00% (w/w) chickpea flour solution was prepared at pH 7.00 using 10 mM sodium phosphate. After overnight stirring at 4℃, 15 mL of the solution was homogenized with a Macro Homogenizer (Omni International, Marietta, Ga, USA) at 7200 rpm for 5 minutes. Foam volume was measured in a 100 mL graduated cylinder at 0 and 30 minutes. Foaming capacity (FC) was calculated according to equations (6):

$$\%FE = \frac{V_{f0}}{V_{li}} \times 100$$

(6) Where V_{li} represents the volume of the initial solution; V_{f0} is the volume of foam immediately after homogenization (Liu et al., 2010).

2.2.8.5. Evaluation of emulsion capacity

The emulsifying properties of chickpea flour were observed by using the method of Setia et al. (2019). In the method, chickpea flour (1.75 g) was dissolved in distilled water (48.25 mL) and adjusted to pH 7.0. Canola oil (75 mL) was added and homogenized at 4-level speed for 1 min by using Omni Macro Homogenizer (Omni International, Marietta, Ga, USA). samples were divided into centrifuge tubes (30 mL each) and tested for stability. The remaining emulsion (60 mL) was heated, cooled, and then centrifuged. Height

measurements were taken for the emulsified layer and the entire solution. The emulsion capacity (EC) was calculated using equation (7):

$$\%EC = \frac{H_{el}}{H_{ee}} \times 100 \quad (7)$$

Where H_{el} is the height of emulsified layer; H_{ee} is the height of the entire emulsion (Setia et al., 2019).

2.2.9. Color evaluation

Color analysis was conducted on flour samples using a chromometer Konica Minolta CR-410 Chroma meter (Konica Minolta, Ramsey, NJ, USA). The L* value represented the lightness of the sample, a* value indicated the degree of greenness/redness, and the b* value reflected the level of blueness/yellowness. The color difference was calculated through the difference in L*, a*, and b* values for 0 days (L^*_1, a^*_1, b^*_1) and 360 days (L^*_2, a^*_2, b^*_2) using the following equation (8):

$$\text{Color difference } (\Delta E) = \sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2} \quad (8)$$

2.2.10. Statistical analysis

There were 6 treatments based on the combination of storage RH and temperature. These included 1) 40% RH at room temperature (21-22℃), 2) 55% RH at room temperature, 3) 65% RH at room temperature, 4) 40% RH at 40 ℃, 5) 55% RH at 40 ℃, and 6) 65% RH at 40℃. Samples were removed at 3 intervals as described earlier. Protein and starch quality evaluations were compared between samples from day 0 and day 360

only for samples stored at 65% RH and 40 °C. All data were analyzed by analysis of variance (three-way ANOVA) and means separation completed using Tukey's test initially and then no differences were observed in most of the tests, so, the LSD test at a 95% confidence level was performed. Time, humidity, and temperature were the independent variables affecting different dependent variables of quality, and their interactions were analyzed.

2.3. Results and Discussion

2.3.1. Nutritional value

Sierra exhibited the highest MC (7.7 ± 0.01%) among all varieties for the zero-day sampling (Table 2.1). The average MC of all varieties increased over time. Various factors, including RH, day, and temperature, significantly correlated to the increase of moisture in varieties (Table 2.2). Notably, Orion had the highest MC (10.3%) under conditions of 65% RH, 21 °C, and 360-day sampling, suggesting that samples at 21 °C absorbed and retained more moisture compared to those at 40 °C. Likely, the seeds stored at the higher temperature were not able to retain the absorbed moisture as well as the seed at lower temperatures. Similar results were observed in chickpeas stored at 55% RH, where significant increases above 13 % MC at 21 °C were observed (Malhotra et al., 2023). Most of the samples kept at high temperatures had less MC compared to those stored at room temperature while all varieties had significantly ($P \leq 0.05$) higher average water content compared to samples from day 0 except Royal stored at 40% and 65%, at temperature of 40 °C for 360 days and samples were kept under 40% and 40 °C for all varieties, which had lower MC compared to zero-day sampling. Frontier stored at 65% and 21 °C had the

highest MC in the 180-day sampling. Overall, stored seed at 55% and 65% RH had higher MC in all samples at 180 and 360 sampling days compared to day 0 samples. The effect of variables was significant ($P \leq 0.05$) for varieties and the interaction of variables was significant for MC in the Crown, Sierra, and Orion (Table 2).

Table 2.1. Effects of the variables (Day, RH, and Temperature) on chemical composition (Moisture, Protein, Total starch, and Fat) of all chickpea varieties (Crown, Royal, Sierra, Orion, and Frontier).

Variety	0-day	180-day					
		21 °C			40 °C		
		40%	55%	65%	40%	55%	65%
Moisture (%)							
Crown	7.0 ± 0.01^{bc}	8.7 ± 0.1^{def}	9.3 ± 0.3^{fg}	9.6 ± 0.1f^{g}	6.9 ± 0.1^{b}	7.9 ± 0.03^{cd}	8.7 ± 0.1^{def}
Royal	7.2 ± 0.01^{abc}	8.6 ± 0^{cde}	9.4 ± 0.5^{de}	9.9 ± 0.3^{e}	6.5 ± 0.6^{a}	7.8 ± 0.3^{abc}	8.2 ± 0.1^{bcd}
Sierra	7.7 ± 0.0^{abcd}	8.7 ± 0.5^{cde}	8.9 ± 0.5^{de}	9.6 ± 0.3^{e}	7.8 ± 0.7^{abc}	6.7 ± 0.6^{ab}	8.3 ± 0.3^{bcde}
Orion	7.1 ± 0.1^{bc}	7.6 ± 0.4^{bcd}	9.3 ± 0.1^{fg}	9.7 ± 0.3^{gh}	6.7 ± 0.1^{ab}	8.0 ± 0.2^{cde}	8.6 ± 0.1^{ef}
Frontier	7.1 ± 0.1^{ab}	8.8 ± 0.1^{def}	9.8 ± 0.1^{fg}	10.1 ± 0.1^{g}	6.8 ± 0.2^{a}	8.3 ± 0.2^{cde}	9.2 ± 0.2^{efg}
Protein (%)							
Crown	20.7 ± 0.4	20.3 ± 0.5	21.5 ± 0.4	21.0 ± 0.4	20.7 ± 0.4	21.2 ± 0.8	21.1 ± 0.4
Royal	20.5 ± 1.0	21.0 ± 1.5	20.8 ± 1.5	20.7 ± 1.1	20.6 ± 1.4	20.8 ± 1.1	21.0 ± 1.4
Sierra	20.4 ± 1.1	21.3 ± 1.9	21.1 ± 0.8	20.8 ± 1.1	20.9 ± 1.1	21.0 ± 1.2	21.4 ± 1.2
Orion	20.7 ± 1.3	20.5 ± 1.5	20.4 ± 1.4	20.5 ± 0.5	20.0 ± 1.2	20.6 ± 1.2	21.1 ± 1.3
Frontier	24.0 ± 0.0^{ab}	24.9 ± 0.5^{bcd}	25.8 ± 0.1^{d}	25.3 ± 0.2^{cd}	24.9 ± 0.2^{bcd}	25.4 ± 0.3^{cd}	25.5 ± 0.2^{cd}
Total starch (%)							
Crown	44.2 ± 0.1^{abc}	44.5 ± 0.8^{bc}	44.7 ± 0.3^{c}	43.5 ± 0.2^{abc}	43.2 ± 2.1^{abc}	41.0 ± 0.7a^{bc}	42.5 ± 1.4^{abc}
Royal	46.5 ± 0^{c}	43.9 ± 1.9^{bc}	44.6 ± 1.8^{c}	42.2 ± 0.9^{b}	43.2 ± 1.6^{bc}	43.3 ± 1.1^{bc}	43.6 ± 0.1^{bc}
Sierra	45.0 ± 0^{b}	44.0 ± 1.2^{ab}	42.6 ± 0^{ab}	44.5 ± 0.4^{ab}	44.7 ± 0.1^{ab}	43.8 ± 0.6^{ab}	42.7 ± 1.9^{ab}
Orion	45.0 ± 0	43.7 ± 1.6	43.7 ± 0.5	45.5 ± 0.5	41.7 ± 1.2	43.7 ± 1.2	45.5 ± 0.6
Frontier	42.4 ± 0^{bc}	41.8 ± 1.1^{abc}	41.2 ± 1.1^{abc}	42.9 ± 1.0^{c}	41.9 ± 0.9^{abc}	42.8 ± 0.1^{c}	41.5 ± 0.1^{abc}
Fat (%)							
Crown	6.5 ± 0.1^{b}	5.4 ± 0.4^{a}	5.5 ± 0.3^{ab}	5.7 ± 0.1^{ab}	5.6 ± 0.1^{ab}	5.8 ± 0.2^{ab}	5.8 ± 0.2^{ab}
Royal	6.3 ± 0.1	5.5 ± 0.4	5.6 ± 0.4	5.6 ± 0.5	5.7 ± 0.4	5.8 ± 0.4	5.9 ± 0.3
Sierra	6.5 ± 0.1	5.9 ± 0.4	5.8 ± 0.2	5.8 ± 0.1	5.8 ± 0.3	5.9 ± 0.2	5.7 ± 0.1
Orion	8.0 ± 0.1^{b}	6.2 ± 0.1^{a}	6.4 ± 0.1^{a}	6.5 ± 0.4^{a}	6.3 ± 0.2^{a}	6.6 ± 0.2^{a}	6.6 ± 0.1^{a}
Frontier	6.6 ± 0^{bcd}	5.9 ± 0.4^{cd}	5.8 ± 0^{d}	5.9 ± 0^{d}	5.9 ± 0^{d}	5.9 ± 0.1^{d}	5.9 ± 0.1^{d}

Variety	0-day	360-day					
		21 °C			40 °C		
		40%	55%	65%	40%	55%	65%
				Moisture (%)			
Crown	7.0 ± 0.01^{bc}	8.3 ± 0.1^{de}	9.4 ± 0.1^{fg}	10.0 ± 0.0^{g}	5.1 ± 0^{a}	8.3 ± 0.7^{de}	8.9 ± 0.1^{ef}
Royal	7.2 ± 0.01^{abc}	8.2 ± 0.1^{bcd}	8.1 ± 0.4^{bcd}	9.5 ± 0.3^{de}	6.6 ± 0.1^{a}	7.7 ± 0.1^{abc}	6.9 ± 0.5^{ab}
Sierra	7.7 ± 0.0^{abcd}	7.6 ± 0.1^{abcd}	8.2 ± 0.7^{bcde}	9.2 ± 0.1^{de}	5.9 ± 0.5^{a}	9.1 ± 0.1^{de}	8.2 ± 0.2^{bcde}
Orion	7.1 ± 0.1^{bc}	7.2 ± 0.1^{bc}	8.5 ± 0.2^{def}	10.3 ± 0.3^{h}	5.9 ± 0.1^{a}	7.9 ± 0.3^{cde}	7.8 ± 0.1^{cde}
Frontier	7.1 ± 0.1^{ab}	7.5 ± 0.5^{abc}	8.8 ± 0.1^{def}	9.8 ± 0.1^{fg}	6.9 ± 0.1^{ab}	7.9 ± 0.4^{bcd}	8.6 ± 0.2^{de}
				Protein (%)			
Crown	20.7 ± 0.4	20.1 ± 0.4	20.1 ± 0.6	20.2 ± 0.8	20.2 ± 0.8	20.3 ± 0.2	20.8 ± 0.1
Royal	20.5 ± 1.0	20.7 ± 1.2	20.6 ± 1.6	20.7 ± 0.8	20.6 ± 0.3	20.3 ± 1.7	20.7 ± 1.0
Sierra	20.4 ± 1.1	20.6 ± 1.1	19.5 ± 1.3	19.7 ± 1.3	18.5 ± 0.1	20.2 ± 0.8	20.4 ± 1.0
Orion	20.7 ± 1.3	19.9 ± 1.4	19.7 ± 0.8	20.0 ± 1.3	20.1 ± 1.4	19.9 ± 1.3	20.0 ± 1.1
Frontier	24.0 ± 0.0^{ab}	23.7 ± 0.4^{a}	24.3 ± 0.3^{abc}	23.8 ± 0.2^{ab}	23.3 ± 0.1^{a}	23.6 ± 0.1^{a}	23.9 ± 0.2^{ab}
				Total starch (%)			
Crown	44.2 ± 0.1^{abc}	40.8 ± 1.0^{abc}	40.1 ± 0.5^{a}	40.4 ± 0.4^{ab}	40.7 ± 0.1^{abc}	41.0 ± 0.7^{abc}	41.3 ± 0.9^{abc}
Royal	46.5 ± 0^{c}	42.8 ± 0.5^{b}	43.6 ± 0.7^{bc}	40.1 ± 0.1^{a}	41.3 ± 2.7^{a}	42.0 ± 2.5^{b}	42.6 ± 0.4^{b}
Sierra	45.0 ± 0^{b}	42.9 ± 1.2^{ab}	44.9 ± 0.7^{b}	43.1 ± 0.1^{ab}	42.4 ± 1.1^{ab}	40.7 ± 1.0^{a}	42.6 ± 0.1^{ab}
Orion	45.0 ± 0	45.0 ± 1.8	44.0 ± 2.2	44.6 ± 0.8	44.4 ± 1.3	42.0 ± 2.4	41.0 ± 2.0
Frontier	42.4 ± 0^{bc}	39.5 ± 0.5^{abc}	40.5 ± 0.2^{abc}	41.2 ± 0.5^{abc}	39.0 ± 0.1^{ab}	38.6 ± 1.3^{a}	39.4 ± 0.6^{abc}
				Fat (%)			
Crown	6.5 ± 0.1^{b}	5.7 ± 0.2^{ab}	5.9 ± 0.2^{ab}	5.9 ± 0.1^{ab}	5.5 ± 0.3^{ab}	5.2 ± 0.1^{ab}	5.4 ± 0.1^{ab}
Royal	6.3 ± 0.1	5.5 ± 0.4	5.8 ± 0.3	5.7 ± 0.4	5.5 ± 0.3	5.7 ± 0.5	5.4 ± 0.4
Sierra	6.5 ± 0.1	5.8 ± 0.3	5.7 ± 0	5.5 ± 0.1	5.7 ± 0.1	5.8 ± 0.1	5.8 ± 0.1
Orion	8.0 ± 0.1^{b}	6.3 ± 0.3^{a}	6.2 ± 0.2^{a}	6.0 ± 0.2^{a}	6.3 ± 0.4^{a}	6.5 ± 0.2^{a}	6.3 ± 0.3^{a}
Frontier	6.6 ± 0^{bcd}	5.3 ± 0.1^{ab}	5.2 ± 0.1^{ab}	5.3 ± 0.1^{abc}	5.1 ± 0.4^{a}	5.7 ± 0^{abcd}	5.7 ± 0.1^{abcd}

Data points represent the mean ± standard deviation of two independent experiments. Different letters indicate statistically significant differences in varieties ($p \leq 0.05$). LSD was performed for the "day," "temperature," and "RH" factors within three-way ANOVA for data (true replicate = 2 and total n = 4). The LSD values are the same for all factors (day, temperature, and RH) because they are calculated based on the overall mean square error from the ANOVA, which is consistent across all factors.

Variations in PC among varieties over the 360-day storage were observed (Table 2.1). Frontier had the highest PC value at 24.0 ± 0.0%, this value was significantly different ($P \leq 0.05$) from the other varieties (PC ranged from 20.5% to 24.0%). Costantini et al. (2021) also reported a consistent PC range (approximately 17-26 %) for different varieties of chickpeas, suggesting a common PC range for this legume. In general, PC decreased slightly during storage, but no significant effects were observed after 360 days for Crown, Royal, Sierra, and Orion compared to 0-day results. The same results were observed for storage temperature and storage length until day 360 for chickpeas, whereas the storage length (540 days) was needed to affect PC (Yeken et al., 2023). The PC decreased for the Frontier variety with effects of the day and RH while other variables did not significantly affect PC. In the storage of chickpeas, Malhotra et al. (2023) observed that RH, temperature, and their interaction had no significant effects on PC.

In contrast to protein, TSC varied among the different varieties. The Royal variety had the highest TSC content at the beginning of the storage period, with a value of 46.5%. However, over 360 days, TSC gradually decreased (Table 2.1). The day significantly ($P \leq 0.05$) affected starch content for all varieties except Orion that did not have a significant decrease ($P \leq 0.05$). Frontier had the lowest TSC content across all sampling days, starting at 42.4% for the zero-day sample and declining to 38.6% for seed stored at 55% RH and 40 ℃, which was significant ($P \leq 0.05$). The percentage point decrease in TSC for all samples after 360 days of storage was 4.1, 6.4, 4.3, 4.0, and 3.8% for Crown, Royal, Sierra, Orion, and Frontier, respectively. This indicates that as the storage duration increased, the TSC content decreased uniformly across all varieties.

Among the varieties examined, Orion consistently had the highest FC, a value of 8.0 % at the beginning of the storage period and 6.3 % (65% RH and 40 °C) at 360 days (Table 2.1). Conversely, Royal had the lowest FC (6.3 ± 0.1%) at the outset of storage and 5.4 ± 0.4% (65% RH and 40 °C) at 360 days. Royal and Sierra had a slight decrease in FC, which was not significant ($P>0.05$) while FC decreased significantly ($P\leq0.05$) in Crown, Orion and Frontier with effects of the storage time (day) variable. As the storage period advanced (Table 2.1), FC consistently decreased in all samples, indicating a clear trend of fat degradation over time, and supporting previously published research (Rajarammanna et al., 2010; Rani et al., 2013). Furthermore, previous researchers found that chickpeas stored at the highest temperature (30 °C) and the highest MC had the highest free fatty acid content (Malhotra et al., 2023), which signifies a breakdown of triacylglycerols. Therefore, the reduction in FC observed in the current study may be due to a breakdown in triacylglycerols

Table 2.2. Effects of variables on chemical, functional, pasting and color properties.

Variety	Test	Day	RH	T	T*RH*Day
Crown	Moisture	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]
	Protein	<0.05[a]	0.48	0.71	0.86
	Total starch	<0.01[a]	0.81	0.25	0.90
	Fat	<0.01[a]	0.06	<0.05 [a]	0.94
	Peak vis	<0.01 [a]	<0.01 [a]	<0.01 [a]	0.47
	Final vis	0.12	<0.01 [a]	0.09	0.83
	Peak temp	<0.01[a]	<0.01[a]	<0.01[a]	0.52
	Gel strength	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]
	WHC	<0.01[a]	0.32	<0.01[a]	0.90
	OHC	<0.01[a]	<0.01[a]	0.18	0.12
	WSI	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]
	WAI	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]
	EC	<0.01[a]	<0.01[a]	<0.01[a]	0.53
	FC	<0.01[a]	0.31	0.29	0.13
	L*	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]
	a*	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]
	b*	<0.01[a]	<0.01[a]	<0.01[a]	0.10
	ΔE	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]
Royal	Moisture	<0.01[a]	<0.01[a]	<0.01[a]	0.06
	Protein	0.17	0.90	0.87	0.99
	Total starch	<0.01[a]	0.49	0.82	0.74
	Fat	<0.05[a]	0.78	0.08	0.98
	Peak vis	<0.01[a]	<0.01[a]	<0.01[a]	<0.05[a]
	Final vis	0.58	0.95	0.11	0.71
	Peak temp	0.36	0.64	<0.05[a]	0.98
	Gel strength	<0.01[a]	<0.05[a]	<0.01[a]	0.62
	WHC	<0.01[a]	0.97	<0.01[a]	0.42
	OHC	0.71	0.07	0.51	0.49
	WSI	<0.01[a]	<0.01[a]	<0.01[a]	0.19
	WAI	<0.01[a]	0.19	<0.05[a]	0.52
	EC	<0.01[a]	0.05	0.84	<0.01[a]
	FC	<0.01[a]	<0.05[a]	0.06	0.36
	L*	<0.05[a]	0.05	<0.05[a]	0.98
	a*	<0.01[a]	0.12	<0.05[a]	0.16
	b*	<0.05[a]	<0.05[a]	0.14	0.72
	ΔE	<0.01[a]	0.28	0.09	0.48
Sierra	Moisture	<0.05[a]	<0.01[a]	<0.01[a]	<0.05[a]
	Protein	<0.05[a]	0.97	0.69	0.63
	Total starch	<0.01[a]	0.70	0.11	0.05
	Fat	<0.05[a]	0.16	<0.05[a]	0.79
	Peak vis	<0.01[a]	0.70	0.50	<0.05[a]
	Final vis	0.06	<0.05[a]	0.13	0.37
	Peak temp	<0.01[a]	0.25	<0.01[a]	0.90
	Gel strength	<0.01[a]	<0.01[a]	<0.01[a]	0.66

	WHC	<0.01[a]	0.62	<0.01[a]	0.81
	OHC	<0.01[a]	0.93	0.47	0.02
	WSI	<0.01[a]	<0.01[a]	<0.01[a]	0.44
	WAI	<0.01[a]	0.21	<0.01[a]	0.96
	EC	<0.01[a]	<0.01[a]	0.74	0.30
	FC	<0.01[a]	0.49	0.11	<0.05[a]
	L*	<0.01[a]	<0.05[a]	<0.05[a]	0.49
	a*	<0.01[a]	0.41	<0.01[a]	0.34
	b*	<0.01[a]	<0.01[a]	0.30	0.22
	ΔE	<0.01[a]	0.55	0.07	0.48
Orion	Moisture	<0.01[a]	<0.01[a]	<0.01[a]	<0.01[a]
	Protein	0.33	0.79	0.67	0.98
	Total starch	0.11	0.73	0.11	0.66
	Fat	<0.01[a]	0.27	0.05	0.61
	Peak vis	<0.01[a]	0.39	0.41	0.41
	Final vis	0.45	<0.05[a]	0.26	0.49
	Peak temp	0.12	0.22	<0.01[a]	0.61
	Gel strength	<0.01[a]	<0.01[a]	<0.01[a]	0.33
	WHC	<0.01[a]	0.06	<0.01[a]	<0.01[a]
	OHC	0.19	<0.01[a]	0.27	<0.05[a]
	WSI	<0.01[a]	<0.05[a]	<0.01[a]	0.59
	WAI	<0.01[a]	0.67	<0.05[a]	0.73
	EC	<0.01[a]	<0.01[a]	0.18	<0.05[a]
	FC	<0.01[a]	0.67	0.97	0.46
	L*	<0.01[a]	<0.01[a]	<0.01[a]	0.15
	a*	<0.01[a]	<0.05[a]	<0.01[a]	<0.05[a]
	b*	0.32	<0.01[a]	0.69	0.22
	ΔE	<0.01[a]	0.09	<0.01[a]	0.16
Frontier	Moisture	<0.01[a]	<0.01[a]	<0.01[a]	0.07
	Protein	<0.01[a]	<0.05[a]	0.20	0.74
	Total starch	<0.01[a]	0.39	0.16	0.32
	Fat	<0.01[a]	0.12	<0.05[a]	0.32
	Peak vis	<0.01[a]	<0.01[a]	<0.01[a]	0.63
	Final vis	<0.01[a]	<0.01[a]	<0.01[a]	0.26
	Peak temp	<0.01[a]	<0.05[a]	<0.01[a]	0.74
	Gel strength	<0.01[a]	0.85	<0.01[a]	0.91
	WHC	<0.01[a]	0.51	<0.01[a]	0.22
	OHC	<0.01[a]	0.27	0.09	0.64
	WSI	<0.01[a]	<0.05[a]	<0.01[a]	0.78
	WAI	<0.01[a]	0.25	<0.01[a]	0.22
	EC	<0.01[a]	<0.01[a]	<0.01[a]	0.43
	FC	<0.01[a]	0.28	0.13	0.67
	L*	<0.05[a]	0.09	<0.01[a]	0.61
	a*	<0.01[a]	<0.05[a]	<0.01[a]	0.44
	b*	0.83	<0.01[a]	0.44	0.19
	ΔE	<0.01[a]	0.17	<0.01[a]	0.52

[a] Means significant effects on responding variables.

2.3.2. Protein quality

2.3.2.1. SDS-PAGE

A change in molecular weight distribution was observed in protein obtained from various chickpea varieties after being stored under conditions of 65% RH and 40°C because overall nutritional changes were greater in these conditions (Figure 2.2). Upon analysis, Frontier, Sierra, Orion, Royal, and Crown at day 0 (F1, S1, O1, R1, and C1) had several bands around 55 kDa. These bands can be attributed to legume proteins, comprising α and β subunits that are crosslinked by disulfide bonds (Gao et al., 2020). Bands observed at around 50 and 37 kDa are significant and noteworthy as they indicate the presence of legumin. Legumin has a notably elevated concentration of sulfur-containing amino acids compared to vicilin. When subjected to reducing conditions, the disulfide (SS) linkages within legumin undergo disruption. This leads to the formation of an acidic subunit, legumin-α, at approximately 40 kDa, and a basic subunit, legumin-β, at around 20 kDa. In contrast, vicilin, lacking cysteine residues and SS linkages, remains unreduced, resulting in discernible bands within the 10-20 kDa range (Chang et al., 2022). However, it is important to note that these major bands disappeared after 360 days of storage under harsh conditions (65% RH and 40°C). This disappearance suggests a potential breakage of disulfide bonds between the α and β subunits of the protein. In addition to the disappearance of the major bands around 37 and 55 kDa, the molecular weight profile of Sierra, Orion, Royal, and Crown at day-360 (S2, O2, R2, and C2) showed the formation of new bands above 100 kDa. Similarly, the Frontier (F2) sample had bands around 75 kDa. These newly formed bands suggest the presence of large protein aggregates. The appearance of these

higher molecular weight bands indicates a potential alteration in the protein structure and stability during storage under the given conditions (Cheng et al., 2023).

The convicillin band completely disappeared from all chickpeas during storage. Notably, samples O2 and R2 exhibited the most significant changes and complete disappearance of bands around 20, 37, and 50 kDa. The unfolded 7S has more active sites and hydrophobic interaction and thus can form larger aggregates with long-term heat induction. Furthermore, the interaction between 7S and 11S is the final stage of aggregation (Zhu et al., 2022). The disappearance of the bands in stored samples indicates that soluble protein in the supernatant decreased, which may be due to the formation of insoluble aggregates (Beck et al., 2017; T. Li et al., 2019).

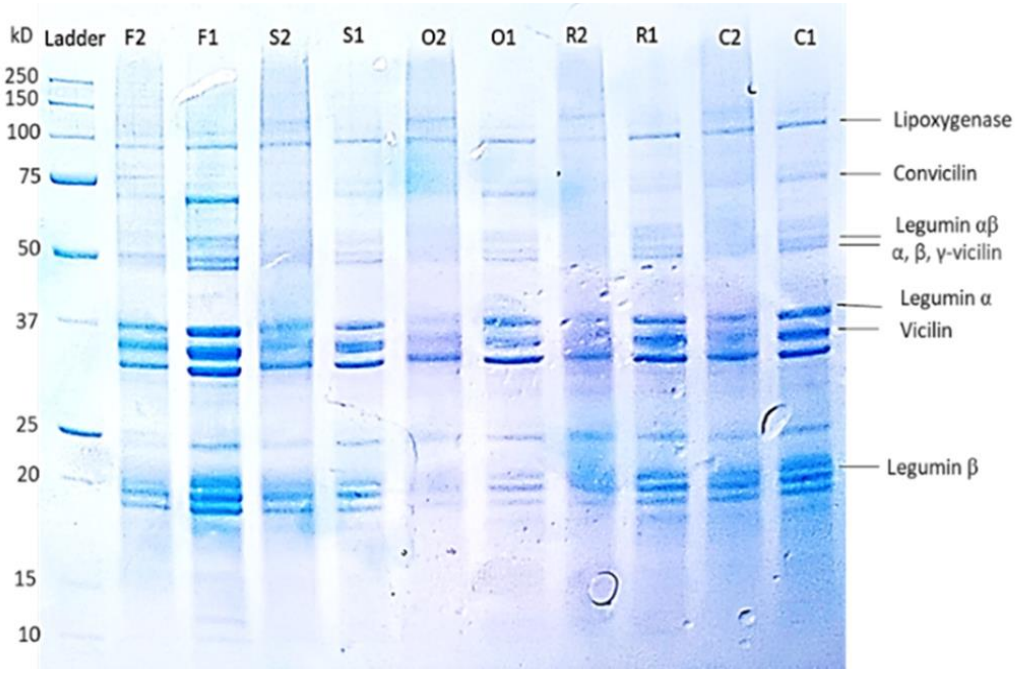

Figure 2.2. Protein bands from SDS-PAGE for isolated protein from chickpea varieties from 0-day and 360-day samples. F1, S1, O1, R1, and C1 (Frontier, Sierra, Orion, Royal, and Crown from 0-day sampling) and F2, S2, O2, R2, and C2 (Frontier, Sierra, Orion, Royal, and Crown from 360-day sampling).

2.3.2.2. FT-IR

The secondary structure of isolated proteins from day-zero samples and day-360 samples under 65% RH and 40 °C were examined using the second derivative of FT-IR spectra (Table 2.3 and Figure 2.3). The identified peaks in the FTIR spectra represented specific structural components such as β-sheet (1638 cm^{-1}), random coil (1643 cm^{-1}), α-helix (1654 cm^{-1}), β-turn (1663 cm^{-1}), anti-parallel β-sheets (1680 cm^{-1}), and aggregates (1691 cm^{-1}).

β-Sheets structure increased in Sierra and Orion while decreasing in other varieties after 360 days of storage. A decrease in β-sheet and an increase in random coil in stored samples isolated proteins exhibited a more flexible structure (Li et al., 2019). For example, Frontier had significant higher content of after 360 days (12.5%) compared to 0 day (8.9%). Royal had the highest β-Sheets content (57.5%) at zero-day sampling compared to the other varieties which decreased after 360 days significantly ($P \leq 0.05$) and reached to 27.4%. Frontier had more β-turn percentage at 0-day sampling, that suggests a rigid and folded globulin structure due to β-turn restricted conformation entropy of the peptide chain (Cui et al., 2020).

High RH increases hydrogen bond distribution and new bonds might develop between protein molecules that lead to protein aggregation (Beck et al., 2017). The impact of various conditions was evident, indicating an increase in the formation of random coils and aggregate structures. Frontier and Crown had significant higher content of aggregates hat increased 6.8% to 9.9% and 6.1% to 11.7%, respectively. These structural changes can positively enhance the functional properties of the protein, such as foaming capacity and foaming stability.

Hydrophobicity increased in 360-day samples for all varieties. Li et al. (2019) reported that protein subjected to heat resulted in hydrophobic group exposure, and hydrophobic aggregate formation occurred while the water-polar group interactions were reinforced, and hydrophobic groups were exposed on the surface of the protein complex molecule.

Table 2.3. The percentage of different secondary structures and hydrophobic groups of protein isolates from different varieties in 0 and 360 days of storage (65% RH, and 40 °C).

Variety	β-Sheets (%)		Random coil (%)		α-Helices (%)		β-Turns (%)	
Day	0	360	0	360	0	360	0	360
Crown	$^{A}50.9\pm 0.1^{b}$	$^{A}42.7\pm 0.1^{a}$	$^{A}9.5\pm 0.1^{c}$	$^{A}16.0\pm 0.4^{a}$	$^{A}8.1\pm 0.1^{c}$	$^{A}9.3\pm 0.1^{c}$	$^{A}19.6\pm 0.2^{b}$	$^{B}12.5\pm 0.1^{c}$
Royal	$^{A}57.5\pm 0.1^{a}$	$^{B}27.4\pm 0.1^{c}$	$^{A}9.6\pm 0.4^{c}$	$^{A}11.2\pm 0.1^{b}$	$^{B}9.1\pm 0.1^{c}$	$^{A}21.0\pm 0.1^{ab}$	$^{B}6.2\pm 0.6^{e}$	$^{A}12.7\pm 0.3^{c}$
Sierra	$^{A}36.8\pm 0.1^{c}$	$^{A}40.7\pm 0.1^{b}$	$^{A}10.3\pm 0.1^{b}$	$^{B}6.1\pm .0.1^{c}$	$^{A}18.2\pm 0.1^{b}$	$^{B}9.2\pm 0.01^{c}$	$^{B}10.6\pm 0.3^{d}$	$^{A}20.4\pm 0.2^{a}$
Orion	$^{A}21.3\pm 0.1^{e}$	$^{A}23.7\pm 0.3^{d}$	$^{A}12.8\pm 0.2^{a}$	$^{B}7.3\pm 0.1^{c}$	$^{A}23.2\pm 0.3^{a}$	$^{A}23.2\pm 0.2^{a}$	$^{A}17.0\pm 0.1^{c}$	$^{B}15.8\pm 0.1^{b}$
Frontier	$^{A}28.2\pm 0.7^{d}$	$^{A}24.7\pm 0.3^{d}$	$^{B}8.9\pm 0.5^{d}$	$^{A}12.5\pm 0.1^{b}$	$^{B}11.7\pm 0.2^{bc}$	$^{A}25.1\pm 0.1^{a}$	$^{A}21.9\pm 0.2^{a}$	$^{A}17.6\pm 0.2^{b}$

Variety	Antiparallel β-sheets (%)		Aggregates (%)		Hydrophobicity (µg/mg)	
Day	0	360	0	360	0	360
Crown	$^{B}5.4\pm 0.1^{d}$	$^{A}7.8\pm 0.1^{d}$	$^{B}6.1\pm 0.3^{d}$	$^{A}11.7\pm 0.1^{c}$	$^{B}31.8\pm 3.5^{a}$	$^{A}36.5\pm 1.88^{a}$
Royal	$^{B}7.1\pm 0.1^{d}$	$^{A}15.1\pm 0.1^{a}$	$^{A}11.9\pm 0.1^{b}$	$^{A}14.0\pm 0.1^{b}$	$^{B}26.6\pm 2.9^{b}$	$^{A}29.6\pm 1.82^{b}$
Sierra	$^{A}16.3\pm 0.1^{a}$	$^{A}14.6\pm 0.1^{b}$	$^{A}8.2\pm 0.5^{c}$	$^{A}9.0\pm 0.1^{d}$	$^{B}14.6\pm 2.2^{d}$	$^{A}30.7\pm 2.23^{c}$
Orion	$^{B}11.3\pm 0.2^{c}$	$^{A}15.7\pm 0.1^{a}$	$^{A}14.3\pm 0.1^{a}$	$^{A}15.1\pm 0.1^{a}$	$^{B}17.2\pm 2.2^{e}$	$^{A}23.5\pm 1.5^{d}$
Frontier	$^{A}13.0\pm 0.5^{b}$	$^{A}10.2\pm 0.1^{c}$	$^{B}6.8\pm 0.8^{d}$	$^{A}9.9\pm 0.1^{d}$	$^{B}22.7\pm 1.9^{c}$	$^{A}24.6\pm 1.92^{d}$

Different lower-case letters indicate significant differences ($p \leq 0.05$) within the same column for 0-day and 360-day samples separately. Different capital letters indicate significant differences ($p \leq 0.05$) between the 0 and 360-day samples for each variety (true replicate = 2 and total n = 4).

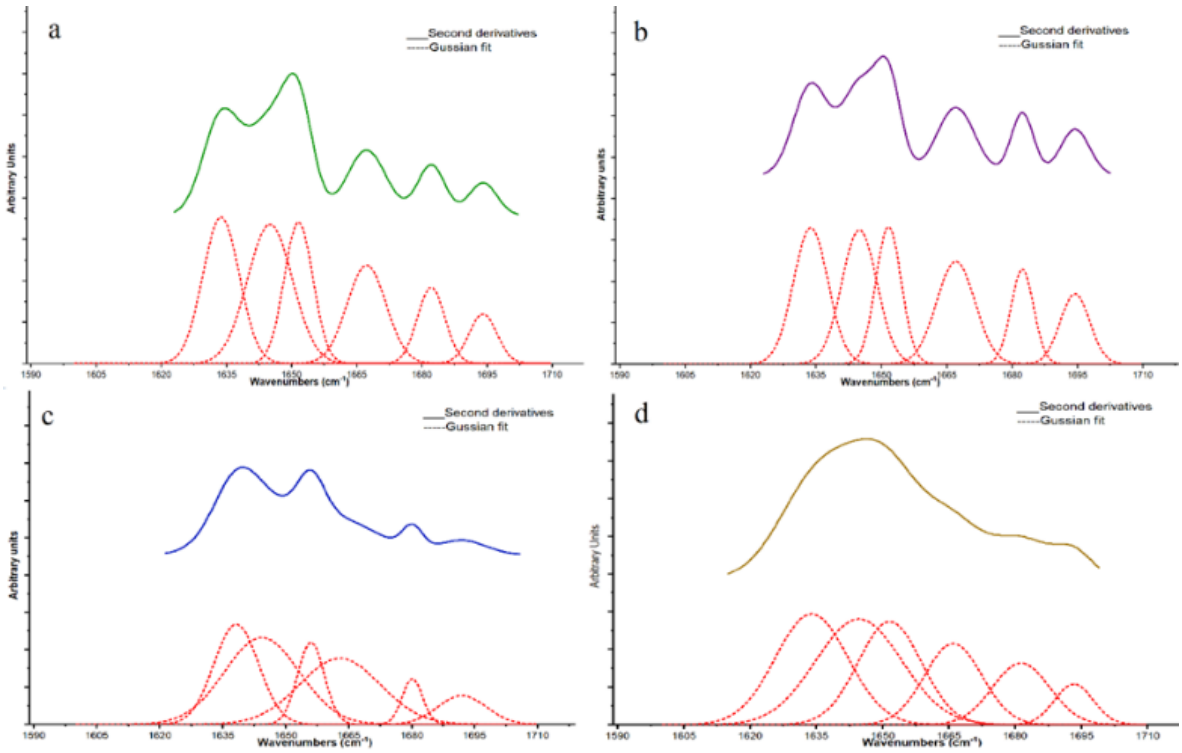

Figure 2.3. FT-IR Second derivative and Gaussian fitted peaks for 2 varieties (a. Orion 0-day, b. Orion 360-day, c. Crown 0-day, and d. Crown-360 day) of chickpea.

2.3.3. Starch

2.3.3.1. SEM

Native (0-day isolated) starch had large oval granules and some spherical granules (Figure 2.4), which are smooth with no evidence of cracks (Beck et al., 2017). After storage, starch granules are enveloped by a protein matrix (Figure 2.3). These results support the hypothesis of the coating effects of protein on starch granules due to the interaction of new protein-protein or protein-starch interactions that occurred during storage under harsh conditions (65% RH and 40°C), which likely contributed to protein aggregation and reduction of protein solubility (Polesi & Sarmento, 2011).

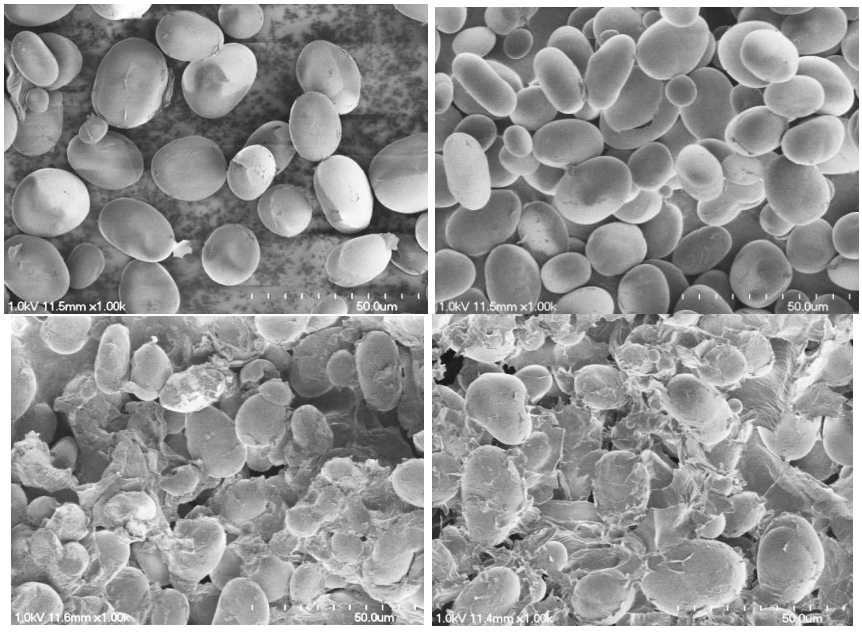

Figure 2.4. Scanning electron microscope images of starch extracted from chickpea flour at 0-day (a) and 360-day (b) samples.

2.3.3.2. Amylose and amylopectin ratio

Amylose and amylopectin of all varieties were analyzed in zero-day and 360-day samples to observe amylose and amylopectin ratio differences among all samples. Amylose percentage in total starch fell between 15 and 38 % (Table 2.4), which is in the range

previously reported (Silvestre-De-León et al., 2020). Amylose content increased in Crown, Sierra, and Frontier by 25.0, 10.4, and 13.7% in 360-day stored chickpeas, respectively, while decreasing in Royal and Orion by 4.0 and 9.9%, respectively. This increase might be due to the debranching of amylopectin and conversion into amylose (Chung et al., 2008). Non-starch carbohydrates increased in all samples by 1.0, 3.8, 1.7, 5.3, and 1.7 percentage points for Crown, Royal, Sierra, Orion, and Frontier, respectively (Table 2.4).

Table 2.4. Starch and non-starch fractions of chickpea varieties of Crown, Royal, Sierra, Orion, and Frontier flours at zero-day and 360-day under 65% RH and 40 ℃.

Variety	Starch						Non-starch carbohydrates	
	Total starch		Amylose/TSC		Amylose/100g*			
	0	360	0	360	0	360	0	360
Crown	44.2 ± 0.10^{d}	41.3 ± 0.9^{b}	38.9 ± 3.8^{a}	63.9 ± 2.4^{a}	16.0 ± 1.6^{a}	26.4 ± 0.1^{a}	18.6 ± 0.4^{c}	19.6 ± 0.7^{b}
Royal	46.5 ± 0.01^{a}	42.6 ± 0.4^{a}	37.0 ± 3.2^{a}	33.0 ± 2.2^{b}	16.7 ± 1.5^{a}	14.1 ± 0.9^{b}	16.6 ± 1.0^{b}	20.4 ± 1.4^{d}
Sierra	45.0 ± 0.01^{b}	42.6 ± 0.1^{a}	15.3 ± 0.3^{c}	25.7 ± 0.5^{c}	6.1 ± 0.1^{c}	11.6 ± 0.2^{c}	17.6 ± 1.0^{e}	19.4 ± 1.3^{a}
Orion	45.0 ± 0.01^{b}	41.0 ± 2.0^{b}	33.2 ± 0.1^{b}	23.4 ± 1.9^{c}	13.8 ± 0.1^{b}	9.7 ± 0.8^{d}	16.4 ± 1.3^{a}	21.7 ± 0.0^{c}
Frontier	42.4 ± 0.01^{c}	39.4 ± 0.6^{c}	17.9 ± 1.9^{c}	31.6 ± 1.6^{b}	7.0 ± 1.1^{c}	12.1 ± 0.6^{c}	17.8 ± 0.0^{d}	19.4 ± 0.1^{b}

All the data were calculated on the dry weight basis. Data points represent the mean ± standard deviation of two independent experiments. Different letters indicate statistically significant differences in varieties ($p \leq 0.05$). Amylose/100 g was calculated based on dry flour weight (true replicate = 2 and total n = 4).

2.3.4. Pasting properties and gel firmness of storage samples

In general, the peak and final viscosities increased across all varieties with storage (Table 2.5). The peak viscosity increased slightly for samples stored under harsh conditions (65% RH and 40 °C) after 360 days compared to samples from day 0. Among the varieties, Sierra exhibited the lowest average peak viscosity (1404 cP) during the initial sampling (0-day), while Royal had the highest average value (1654 cP). The peak viscosity changed significantly under the effects of day for varieties ($P \leq 0.05$) while RH and T variables were significantly effective in increasing peak viscosity for Royal and Frontier. Peak viscosity is the ability to swell and indicates granule uptake of water. Less water uptake results in less swelling. If the water-AP H-bonding occurs more readily, more swelling happens.

Royal had the highest final viscosity (2326 cP), while Frontier from the 0-day sampling had the lowest (1736 cP). The final viscosity increased significantly ($P \leq 0.05$) for cultivars with the effect of RH. Furthermore, final viscosity of the Frontier variety increased with the effect of all variables significantly ($P \leq 0.05$). Bucsella et al., (2016) revealed that a rise in viscosity values of wheat flour stored at high RH and high temperature was correlated with protein denaturation, protein changes, and swelling of starch granules. Interaction of the denatured protein with starch granules and crosslinked protein networks leads to exposure of hydrophobic groups, which facilitates hydrophobic interactions and formation of aggregates (Table 2.3). In turn, the aggregate of starch and protein formation during cooling leads to an increase in resistance to shear force and higher FV (Bucsella et al., 2016; Y. Ma et al., 2021).

Peak temperature decreased significantly ($P \leq 0.05$) for all cultivars with the effects of storage temperature (Table 2.2). Furthermore, in addition to the storage temperature effect, RH and Day had significant ($P \leq 0.05$) effects on peak temperature in Crown and Frontier cultivars. Frontier had the highest peak temperature across all sampling days, which could be due to starch composition and granule structure. The samples in 65% RH and 40 °C at 360 days of storage had the lowest peak temperature in all cultivars.

Gel firmness is mainly caused by retrogradation relating to the re-crystallization of the amylose and amylopectin structure. Furthermore, gel firmness decreased in all samples significantly ($P \leq 0.05$) with the effects of all variables during the storage, with Orion having the highest gel firmness and Frontier having the lowest gel firmness at the zero-day sampling (Table 2.5). The lower gel firmness of the Frontier cultivar compared to other cultivars is due to low TSC. The lower TSC and the higher amylopectin content in Sierra likely account for the lower gel firmness compared to the Royal cultivar. Amylopectin is a highly branched molecule and prohibits the mobility of water molecules (Cornejo-Ramírez et al., 2018). This reduced water mobility results in a gel that is less firm because the amylopectin retains the trapped water and does not readily reassociate into crystalline networks. Furthermore, the formation of protein aggregates in stored samples may restrict particle mobility because protein aggregates tend to form shells or films at the granule exterior wall. This wall acts as a barrier to water migration and crystalline melting, amylose leaching, and swelling of granules and the formation of softer gels for samples stored for 360 days (Kumar et al., 2022). Also, aggregate content has been linked to less firm gels (Beck et al., 2017). Thus, the increase in aggregate content in all varieties in 360-day samples likely leads to an increase in viscosities and a decrease in gel firmness.

Table 2.5. Effects of the variables (Day, RH, and Temperature) on pasting and final viscosity, peak temperature, and gel firmness of all chickpea varieties (Crown, Royal, Sierra, Orion, and Frontier flours at zero-day and 360-day under 65% RH and 40 °C.

Variety	0-day	180-day					
		21 °C			40 °C		
		40%	55%	65%	40%	55%	65%
				Peak Viscosity (cP)			
Crown	1403 ± 50^{a}	1233 ± 71^{a}	1317 ± 99^{a}	1500 ± 54^{ab}	1434 ± 99^{a}	2094 ± 98^{abc}	2694 ± 90^{bc}
Royal	1654 ± 25^{ab}	1678 ± 53^{ab}	1633 ± 19^{ab}	1353 ± 98^{a}	1959 ± 40^{bc}	2007 ± 43^{bc}	2545 ± 99^{c}
Sierra	1404 ± 28^{a}	1578 ± 47^{a}	1509 ± 35^{a}	1510 ± 24^{a}	1532 ± 06^{a}	2042 ± 30^{a}	2492 ± 96^{b}
Orion	1488 ± 54^{a}	1616 ± 46^{ab}	1649 ± 28^{ab}	1639 ± 10^{ab}	1653 ± 26^{ab}	2974 ± 08^{abcd}	2928 ± 10^{cd}
Frontier	1424 ± 13^{a}	1420 ± 11^{a}	1609 ± 19^{ab}	1632 ± 33^{ab}	1719 ± 65^{abcd}	1848 ± 44^{bcde}	2305 ± 31^{cde}
				Final Viscosity (cP)			
Crown	2012 ± 41^{ab}	1706 ± 37^{ab}	1904 ± 90^{ab}	2112 ± 67^{ab}	1610 ± 76^{a}	2061 ± 89^{ab}	2389 ± 90^{b}
Royal	2109 ± 52	2315 ± 02	2636 ± 3	2557 ± 93	2132 ± 17	2645 ± 15	2646 ± 22
Sierra	2139 ± 12^{a}	2245 ± 42^{a}	2060 ± 06^{a}	2329 ± 99^{a}	2091 ± 92^{a}	2195 ± 01^{ab}	2699 ± 21^{ab}
Orion	2326 ± 82	2408 ± 99	2122 ± 02	2296 ± 88	2144 ± 96	2266 ± 06	2706 ± 41
Frontier	1736 ± 59^{ab}	1716 ± 28^{a}	1931 ± 30^{abcd}	1822 ± 21^{abcd}	1968 ± 05^{bcde}	2071 ± 81^{def}	2247 ± 20^{f}
				Peak Temperature (°C)			
Crown	76.71 ± 0.1^{cd}	76.45 ± 0.2^{cd}	76.51 ± 0.2^{cd}	76.3 ± 0.1^{cd}	75.86 ± 0.0^{cd}	74.71 ± 0.4^{abc}	73.66 ± 1.0^{ab}
Royal	75.00 ± 0.1	75.66 ± 1.0	75.22 ± 1	75.48 ± 1.1	73.91 ± 0.4	73.9 ± 0.8	73.94 ± 1.2
Sierra	76.69 ± 0.1^{c}	75.91 ± 0.8^{abc}	76.29 ± 0.4^{bc}	75.73 ± 0.2^{abc}	74.93 ± 0.7^{abc}	74.54 ± 0.6^{abc}	73.44 ± 1.3^{ab}
Orion	74.50 ± 0.0	75.10 ± 0.1	74.92 ± 0.2	74.54 ± 0.2	73.96 ± 0.4	74.56 ± 0.6	73.09 ± 0.9
Frontier	78.32 ± 0.1^{de}	78.51 ± 0.2^{e}	78.26 ± 0.0^{de}	77.86 ± 0.0^{de}	77.14 ± 0.4^{bcde}	75.7 ± 0.6^{abc}	75.91 ± 0.1^{abc}
				Gel Strength (g/s)			
Crown	238 ± 1^{c}	250 ± 1.0^{c}	239 ± 8.0^{c}	191 ± 13^{bc}	191 ± 13^{bc}	39 ± 4^{a}	31 ± 2^{a}
Royal	319 ± 1^{b}	294 ± 8.0^{b}	297 ± 34^{b}	238 ± 41^{ab}	152 ± 2^{ab}	135 ± 6^{ab}	59 ± 10^{a}
Sierra	239 ± 5de	270 ± 37^{e}	241 ± 54de	233 ± 36de	170 ± 34^{bcde}	65 ± 3^{abc}	38 ± 7^{ab}
Orion	253 ± 4^{c}	286 ± 27^{c}	261 ± 30^{c}	250 ± 21^{c}	195 ± 20^{bc}	68 ± 5^{a}	42 ± 7^{a}
Frontier	111 ± 2^{d}	46 ± 3^{abc}	44 ± 1^{abc}	42 ± 2^{abc}	30 ± 5^{ab}	27 ± 1^{a}	29 ± 2^{ab}

Variety	0-day	360-day					
		21 °C			40 °C		
		40%	55%	65%	40%	55%	65%
Peak Viscosity (cP)							
Crown	1403 ± 50^{a}	1288 ± 29^{a}	1329 ± 11^{a}	1433 ± 97^{a}	1686 ± 30^{abc}	2212 ± 40^{abc}	3132 ± 99^{c}
Royal	1654 ± 25^{ab}	1356 ± 9^{a}	1619 ± 37^{ab}	1739 ± 30^{ab}	2121 ± 92^{bc}	2558 ± 50^{c}	3363 ± 89^{d}
Sierra	1404 ± 28^{a}	1331 ± 10^{a}	1446 ± 56^{a}	1451 ± 15^{a}	1505 ± 50^{a}	3095 ± 28^{b}	3119 ± 95^{b}
Orion	1488 ± 54^{a}	1502 ± 05^{a}	1540 ± 88^{ab}	1679 ± 66^{ab}	1803 ± 78^{abc}	3176 ± 16^{bcd}	3501 ± 98^{d}
Frontier	1424 ± 13^{a}	1415 ± 09^{a}	1487 ± 35^{ab}	1872 ± 10^{abcd}	1912 ± 98^{bcde}	2741 ± 39de	2749 ± 39^{e}
Final Viscosity (cP)							
Crown	2012 ± 41^{ab}	1775 ± 3^{ab}	1966 ± 26^{ab}	2113 ± 27^{ab}	2068 ± 17^{ab}	2280 ± 15^{ab}	2346 ± 47^{ab}
Royal	2109 ± 52	2003 ± 26	2266 ± 72	2659 ± 03	2615 ± 08	2597 ± 77	3228 ± 83
Sierra	2139 ± 12^{a}	1997 ± 99^{a}	2109 ± 99^{a}	2134 ± 82^{a}	2261 ± 30^{a}	2574 ± 96^{ab}	2884 ± 98^{b}
Orion	2326 ± 82	1943 ± 03	2316 ± 03	2369 ± 99	2055 ± 99	2345 ± 80	2695 ± 45
Frontier	1736 ± 59^{ab}	1690 ± 17^{a}	1803 ± 51^{abc}	2055 ± ^{96cdef}	2056 ± 32^{cdef}	2215 ± 89^{ef}	2523 ± 32^{d}
Peak Temperature (°C)							
Crown	76.71 ± 0.1^{cd}	77.15 ± 0.5^{d}	76.30 ± 0.4^{cd}	76.23 ± 0.4^{cd}	75.55 ± 0.4^{abcd}	73.93 ± 0.4^{ab}	73.48 ± 0.8^{a}
Royal	75.00 ± 0.1	75.88 ± 0.8	74.68 ± 1.2	74.7 ± 0.4	73.93 ± 1.3	73.05 ± 1.3	73.85 ± 0.3
Sierra	76.69 ± 0.1^{c}	76.65 ± 0.8^{c}	75.53 ± 0.4^{abc}	75.9 ± 0.8^{abc}	73.88 ± 0.4^{abc}	73.05 ± 0.5^{a}	73.10 ± 0.5^{a}
Orion	74.50 ± 0.0	74.73 ± 0.5	75.13 ± 0.1	74.68 ± 0.4	73.45 ± 0.8	73.08 ± 0.5	73.08 ± 0.4
Frontier	78.32 ± 0.1^{de}	78.3 ± 0.9^{de}	78.3 ± 0.1^{de}	77.86 ± 0.0^{cde}	76.33 ± 0.4^{abcde}	74.70 ± 1.3^{a}	74.78 ± 0.4^{ab}
Gel Strength (g/s)							
Crown	238 ± 1^{c}	191 ± 18^{bc}	168 ± 14^{b}	186 ± 29^{bc}	126 ± 23^{b}	29 ± 00^{a}	30 ± 4^{a}
Royal	319 ± 1^{b}	257 ± 29^{ab}	264 ± 27^{ab}	238 ± 40^{ab}	201 ± 90^{ab}	48 ± 6^{a}	44 ± 8^{a}
Sierra	239 ± 5de	215 ± 22^{cde}	195 ± 26^{cde}	195 ± 15de	113 ± 27^{abcd}	31 ± 2^{a}	34 ± 1^{ab}
Orion	253 ± 4^{c}	244 ± 33^{c}	225 ± 34^{bc}	225 ± 9^{bc}	117 ± 30^{ab}	39 ± 10^{a}	36 ± 3^{a}
Frontier	111 ± 2^{d}	60 ± 16^{c}	57 ± 2^{bc}	52 ± 5^{abc}	27 ± 2^{a}	27 ± 0^{a}	25 ± 4^{ab}

Different letters indicate statistically significant differences in varieties ($p \leq 0.05$). LSD was performed for the "day," "temperature," and "RH" factors within three-way ANOVA for data (true replicate = 2 and total n = 4). The LSD values are the same for all factors (day, temperature, and RH) because they are calculated based on the overall mean square error from the ANOVA, which is consistent across all factors. Values with lowercase letters means no significant difference among treatments.

2.3.5. Functional properties

Water-holding capacity (WHC) of flour is an important factor showing its ability to hold water against gravity (Kinsella, 1979). The WHC was affected by day and temperature factors in Crown, Royal, Sierra, Orion, and Frontier (Table 2.2). The WHC decreased for Crown, Royal, Sierra, and Orion and increased for Frontier after 360 days of storage under harsh conditions. Frontier stored 360 days at 40 ℃ and 65% RH had the highest WHC (0.95 ± 0.06 g/g), which was 20 percentage points higher than the initial WHC observed in Frontier at zero-day sampling. Sierra had the highest initial WHC (0.93 ± 0.04 g/g) but was 13 percentage points lower than the Sierra sample from 360-day storage at 40 ℃ and 65% RH. The highest WHC (1.06 ± 0.02 g/g) was observed in the Frontier sample from the 180-day sampling that had been stored at 55% RH and 40 ℃. Frontier had the highest protein content, and this result supports previous studies where Kabuli chickpea had increased WHC after boiling, which the authors attributed to protein denaturation (Ma et al., 2011).As discussed previously that protein structure changed, and hydrophobicity increased after the storage period under harsh conditions which might be the reason decrease in WHC for Crown, Royal, Sierra, and Orion.

Oil Holding Capacity (OHC) did not change significantly for Royal with the effects of all variables. Sierra with the highest initial OHC, which decreased significantly from 0.47 ± 0 to 0.34 ± 0.05 g/g after the 360-day storage period under effects of the day. Since OHC depends on the hydrophobicity of protein content and hydrophobic amino acids (Elkhalifa & Bernhardt, 2010), OHC enhancement in Frontier could be attributed to an

increase in the exposed amino acids with non-polar groups that can then interact with the oil.

The WAI and WSI are two important measures that indicate the weight gained as water when flour is dispersed in neutral pH buffer and the weight of material that remains in the supernatant, respectively. The WAI and WSI values are dependent on the hydrophilic structures of proteins and carbohydrates drive the interactions between flour and water or neutral buffer. The results indicated an increase in WAI and a decrease in WSI for all cultivar occurred. However, day and temperature affected WAI while the effects of day, temperature, and RH variables all impacted WSI (Table 2.2). The highest initial WAI was determined for Orion (1.86 ± 0 g/g) which increased to 2.62 ± 0.06 g/g after 360 days of seed storage at 65% RH and 40 °C. WAI was reported from 1.24 to 1.39 g/g for pea and pigeon pea (Maninder et al., 2007) and 1.33 to 1.47 g/g for chickpeas (Kaur & Singh, 2005). Thus, the data for day 0 agrees with literature values and supports changes in the chickpea during storage that leads to higher water absorption. The highest initial WSI was in the Crown cultivar, which decreased from 0.36 ± 0 g/g to 0.15 ± 0 g/g and suggests that this might be due to changes in starch and protein structure (i.e., aggregate formation as previously described) during storage, resulting in less material being dispersible or soluble (Xiao et al., 2015).

Only small differences in EC values were observed among the chickpea cultivars. The lowest EC was 35.9% for the Orion cultivar while the highest 37.5% for Royal during the initial 0-day sampling (Table 2.6). However, as the storage time progressed, Frontier had the lowest EC (28%) among samples stored 360 days at 55% RH and 21 °C. In general, EC increased for Crown, Sierra, Orion, and Frontier cultivars from zero to 360 days of

storage. The EC decreased significantly in the Royal cultivar under the effect of RH. The different protein structures as well as other components such as carbohydrates may be the reason for different EC changes among cultivars of chickpea (Kaur & Singh, 2005). Royal and Sierra had the highest FC for samples on 0-day (266 and 333%, respectively). However, FC decreased significantly for all chickpea samples from 0 to 360 days of storage (Table 2.6). The reduction in FC was enhanced by high temperature (40 °C) and relative humidity (65%). This might be due to protein aggregation and reduction of solubility of protein as well as exposure to hydrophobic groups (Polesi & Sarmento, 2011; Silvestre-De-León et al., 2020). Royal and Sierra had the highest FC for samples on 0-day (266 and 333%, respectively).

Table 2.6. Effects of the variables (day, RH, and temperature) on functional properties (WHC, OHC, WSI, WAI, foaming and emulsion capacity of all chickpea varieties (Crown, Royal, Sierra, Orion, and Frontier).

Variety	Day-0	Day-180					
		21℃			40℃		
		40	55	65	40	55	65
				WHC (g/g)			
Crown	0.84 ± 0.01^{ab}	0.72 ± 0.01^{a}	0.75 ± 0.03^{a}	0.79 ± 0.09^{ab}	0.84 ± 0^{ab}	0.89 ± 0.02^{ab}	0.95 ± 0.02^{b}
Royal	0.76 ± 0^{abc}	0.75 ± 0.02^{abc}	0.72 ± 0.01^{ab}	0.69 ± 0.02^{ab}	0.88 ± 0.06^{abc}	0.88 ± 0.04^{bc}	0.93 ± 0.09^{c}
Sierra	0.97 ± 0.04^{ab}	0.72 ± 0.03^{a}	0.74 ± 0.10^{ab}	0.76 ± 0.07^{ab}	0.85 ± 0.05^{ab}	0.94 ± 0.12^{ab}	$1.03 \pm 0b$
Orion	0.71 ± 0.02^{abc}	0.83 ± 0.01^{cd}	0.71 ± 0.05^{abc}	0.86 ± 0.01^{d}	0.91 ± 0.04^{d}	0.94 ± 0.07^{de}	1.07 ± 0.03^{e}
Frontier	0.79 ± 0.02^{a}	0.88 ± 0.05^{abc}	0.83 ± 0.01^{ab}	0.90 ± 0.04^{abc}	0.96 ± 0^{abc}	1.06 ± 0.02^{bc}	1.07 ± 0.01^{c}
Correlation with protein					**R = 0.48**		
Correlation with starch					**R = -0.16**		
				OHC (g/g)			
Crown	0.35 ± 0.01^{ab}	0.38 ± 0.01^{ab}	0.39 ± 0.01^{ab}	0.36 ± 0.02^{ab}	0.40 ± 0.4^{ab}	0.40 ± 0.02^{ab}	0.38 ± 0.02^{ab}
Royal	0.39 ± 0	0.36 ± 0.01	0.41 ± 0.03	0.37 ± 0.0^{3}	0.42 ± 0.01	0.41 ± 0.02	0.38 ± 0.01
Sierra	0.47 ± 0^{c}	0.38 ± 0.01^{ab}	0.35 ± 0.01^{ab}	0.33 ± 0.01^{a}	0.36 ± 0.03^{ab}	0.36 ± 0.02^{ab}	$0.32 \pm 0.01a$
Orion	0.36 ± 0^{ab}	0.37 ± 0.01^{ab}	0.31 ± 0^{a}	0.34 ± 0.01^{ab}	0.35 ± 0.01^{ab}	0.36 ± 0^{ab}	0.34 ± 0.01^{ab}
Frontier	0.34 ± 0^{a}	0.42 ± 0.02^{abc}	0.38 ± 0.01^{abc}	0.36 ± 0.02^{ab}	0.42 ± 0.01^{abc}	0.39 ± 0.01^{abc}	0.37 ± 0.03^{abc}
Correlation with protein					**R = 0.47**		
Correlation with starch					**R = -0.47**		
				WAI (g/g)			
Crown	1.70 ± 0^{a}	2.11 ± 0.1^{bcd}	2.05 ± 0.05^{bc}	2.14 ± 0.03^{bcd}	2.26 ± 0.02^{bcd}	2.28 ± 0.04^{d}	2.29 ± 0.02^{d}
Royal	1.82 ± 0^{a}	2.14 ± 0.02^{ab}	2.05 ± 0.01^{ab}	1.99 ± 0.03^{ab}	2.23 ± 0.01^{b}	2.18 ± 0.08^{ab}	2.17 ± 0.02^{ab}
Sierra	1.37 ± 0^{a}	1.96 ± 0.06^{bc}	1.93 ± 0.01^{bc}	1.90 ± 0.08^{b}	2.08 ± 0.02^{bcd}	2.11 ± 0.12^{bcd}	2.12 ± 0.14^{bcd}
Orion	$1.86 \pm {}^{0}a$	2.14 ± 0.23^{ab}	2.10 ± 0.11^{ab}	2.17 ± 0.01^{ab}	2.25 ± 0.01^{ab}	2.15 ± 0.04^{ab}	2.20 ± 0.04^{ab}
Frontier	1.60 ± 0^{a}	2.13 ± 0.05^{bc}	2.14 ± 0.01^{bc}	2.14 ± 0.03^{bc}	2.19 ± 0.01^{bc}	2.09 ± 0.05^{b}	2.18 ± 0.02^{bc}
Correlation with protein					**R = 0.03**		
Correlation with starch					**R = -0.33**		

				WSI (g/g)			
Crown	0.36 ± 0^{f}	0.29 ± 0.02^{de}	0.28 ± 0.01^{de}	0.28 ± 0.01^{de}	0.25 ± 0.02^{bcd}	0.23 ± 0.01^{bc}	0.23 ± 0^{bc}
Royal	0.26 ± 0^{cdef}	0.28 ± 0.01^{ef}	$0.28 \pm 0.01d^{ef}$	0.28 ± 0^{def}	0.24 ± 0.01^{bcde}	0.24 ± 0.01^{bcd}	0.23 ± 0.01^{bc}
Sierra	0.31 ± 0^{f}	0.30 ± 0.01^{ef}	0.30 ± 0.01ef	0.30 ± 0.01^{ef}	0.27 ± 0^{cdef}	0.24 ± 0.01^{cde}	0.22 ± 0.01^{abc}
Orion	0.25 ± 0^{c}	0.27 ± 0.03^{c}	0.25 ± 0.02^{c}	0.25 ± 0.01^{c}	0.23 ± 0.02^{bc}	0.23 ± 0.02^{bc}	0.22 ± 0.01^{bc}
Frontier	0.27 ± 0^{h}	0.26 ± 0^{gh}	0.25 ± 0^{fgh}	0.24 ± 0.01^{efgh}	0.24 ± 0.01^{defg}	0.21 ± 0.02^{bcde}	0.21 ± 0^{bcde}
Correlation with protein					**R = -0.19**		
Correlation with starch					**R = 0.28**		
				Emulsion Capacity (%)			
Crown	36.94 ± 1.9^{ab}	36.89 ± 1^{bcde}	35.08 ± 0^{abc}	36.38 ± 0.5^{abcd}	39.24 ± 0.5^{de}	37.38 ± 0.5^{bcde}	38.62 ± 1.2^{bcde}
Royal	37.47 ± 0.8^{d}	37.15 ± 0.3^{cd}	35.50 ± 0.4^{bcd}	34.21 ± 0.9^{ab}	35.77 ± 0.8^{bcd}	34.14 ± 1.2^{ab}	36.84 ± 0^{cd}
Sierra	36.84 ± 0^{ab}	39.67 ± 1.3^{bc}	37.96 ± 0.5^{ab}	35.0 ± 0^{a}	37.96 ± 0.5^{ab}	38.42 ± 0.5^{ab}	36.84 ± 0^{ab}
Orion	35.92 ± 0.9	40.46 ± 2.5^{c}	37.86 ± 1.4^{c}	33.28 ± 0^{a}	36.32 ± 1.3^{abc}	38.47 ± 2.2^{c}	34.16 ± 0.4^{ab}
Frontier	36.84 ± 0^{cd}	37.47 ± 0^{d}	30.99 ± 1.6^{ab}	31.40 ± 3.4^{ab}	38.48 ± 0.7^{d}	34.21 ± 1.2^{bcd}	32.97 ± 3.4^{abc}
Correlation with protein					**R = -0.36**		
Correlation with starch					**R = 0.31**		
				Foaming Capacity (%)			
Crown	220 ± 0^{c}	95.0 ± 9^{ab}	83.3 ± 33^{ab}	116.3 ± 37^{abc}	65.0 ± 45^{ab}	76.66 ± 10^{ab}	66.66 ± 6^{ab}
Royal	266.66 ± 20e	106.66 ± 52^{abc}	156.67 ± 20^{cd}	106.66 ± 3^{abc}	156.67 ± 20^{bc}	71.67 ± 6^{abc}	78.33 ± 13^{abc}
Sierra	333.33 ± 17^{c}	138.33 ± 25^{b}	100 ± 0^{ab}	71.67 ± 35^{ab}	81.67 ± 27^{ab}	66.67 ± 3^{ab}	70 ± 20^{ab}
Orion	203.33 ± 27^{c}	58.33 ± 5^{a}	76.67 ± 23^{a}	43.33 ± 3^{a}	63.33 ± 10^{a}	41.67 ± 8^{a}	53.33 ± 10^{a}
Frontier	190 ± 10^{c}	71.67 ± 15^{ab}	101.67 ± 8^{b}	71.67 ± 18^{ab}	58.33 ± 35^{ab}	98.33 ± 5^{b}	66.67 ± 17^{ab}
Correlation with protein					**R = -0.05**		
Correlation with starch					**R = 0.37**		

Variety	Day-0	Day-360					
		21℃			40℃		
		40	55	65	40	55	65
				WHC (g/g)			
Crown	0.84 ± 0.01^{ab}	0.76 ± 0.01^{a}	0.71 ± 0^{a}	0.72 ± 0.02^{a}	0.75 ± 0.06^{a}	0.77 ± 0.01^{a}	0.73 ± 0.01^{a}
Royal	0.76 ± 0^{abc}	0.70 ± 0.09^{ab}	0.68 ± 0.02^{a}	0.71 ± 0.01^{ab}	0.77 ± 0^{abc}	0.81 ± 0.04^{abc}	0.74 ± 0.04^{abc}
Sierra	0.97 ± 0.04^{ab}	0.73 ± 0.06^{a}	0.70 ± 0.01^{a}	0.71 ± 0.04^{a}	0.78 ± 0.04^{ab}	0.78 ± 0.05^{ab}	0.81 ± 0.02^{ab}
Orion	0.71 ± 0.02^{abc}	0.81 ± 0.01	0.60 ± 0.01^{a}	0.68 ± 0.01^{ab}	0.83 ± 0^{cd}	0.90 ± 0.01^{d}	0.60 ± 0.01^{a}
Frontier	0.79 ± 0.02^{a}	0.81 ± 0.05^{a}	0.87 ± 0.06^{abc}	0.75 ± 0.06^{a}	0.86 ± 0.09^{abc}	0.87 ± 0.06^{abc}	0.95 ± 0.06^{abc}
Correlation with protein					**R = 0.48**		
Correlation with starch					**R = -0.16**		
				OHC (g/g)			
Crown	0.35 ± 0.01^{ab}	0.38 ± 0.04^{ab}	0.37 ± 0.02^{ab}	0.33 ± 0.02^{a}	0.34 ± 0.01^{a}	0.43 ± 0.02^{b}	0.34 ± 0.01^{a}
Royal	0.39 ± 0	0.35 ± 0.02	0.44 ± 0.05	0.39 ± 0.1	0.42 ± 0.04	0.41 ± 0.06	0.36 ± 0
Sierra	0.47 ± 0^{c}	0.33 ± 0.01^{a}	0.36 ± 0.02^{ab}	0.43 ± 0.02^{bc}	0.38 ± 0^{abc}	0.36 ± 0.02^{ab}	0.34 ± 0.05^{a}
Orion	0.36 ± 0^{ab}	0.38 ± 0.01^{ab}	0.37 ± 0.01^{ab}	0.31 ± 0.03^{a}	0.42 ± 0.02^{b}	0.32 ± 0.04^{a}	0.35 ± 0.05^{ab}
Frontier	0.34 ± 0^{a}	0.42 ± 0.02^{abc}	0.40 ± 0.01^{abc}	0.39 ± 0.05^{abc}	0.42 ± 0.01^{abc}	0.44 ± 0^{bc}	0.46 ± 0^{c}
Correlation with protein					**R = 0.47**		
Correlation with starch					**R = -0.47**		
				WAI (g/g)			
Crown	1.70 ± 0^{a}	2.05 ± 0.05^{b}	2.09 ± 0.02^{bcd}	2.08 ± 0.01^{bcd}	2.24 ± 0.05^{bcd}	2.27 ± 0.04^{cd}	2.88 ± 0.05^{e}
Royal	1.82 ± 0^{a}	2.15 ± 0.03^{ab}	2.32 ± 0^{bc}	2.35 ± 0.12^{bc}	2.23 ± 0.05^{b}	2.33 ± 0.05^{bc}	2.67 ± 0.18^{c}
Sierra	1.37 ± 0^{a}	2.15 ± 0.09^{bcd}	2.33 ± 0.01^{bcd}	2.00 ± 0.26^{bc}	2.36 ± 0.08^{cd}	2.54 ± 0.26^{d}	2.34 ± 0.18^{bcd}
Orion	$1.86 \pm {}^{0}a$	2.10 ± 0.04^{ab}	2.12 ± 0.02^{ab}	2.14 ± 0.05^{ab}	2.24 ± 0.05^{ab}	2.60 ± 0.56^{b}	2.62 ± 0.06^{b}
Frontier	1.60 ± 0^{a}	2.47 ± 0.05^{e}	2.38 ± 0.03^{de}	2.37 ± 0.02^{de}	2.25 ± 0.01^{bcd}	2.31 ± 0.03^{cd}	2.24 ± 0.03^{bcd}
Correlation with protein					**R = 0.03**		
Correlation with starch					**R = -0.33**		
				WSI (g/g)			
Crown	0.36 ± 0^{f}	0.26 ± 0.03^{ef}	0.28 ± 0.01^{de}	0.29 ± 0^{de}	0.26 ± 0.02^{cd}	0.20 ± 0.04^{b}	0.15 ± 0^{a}
Royal	0.26 ± 0^{cdef}	0.30 ± 0^{f}	0.24 ± 0.01^{bcde}	0.24 ± 0.01^{bcde}	0.23 ± 0.01^{bcd}	0.20 ± 0.02^{b}	0.15 ± 0.02^{a}
Sierra	0.31 ± 0^{f}	0.29 ± 0.01^{ef}	0.26 ± 0.02^{cdef}	0.27 ± 0.01^{def}	0.23 ± 0.01^{bcd}	0.17 ± 0.02^{a}	0.17 ± 0.01^{ab}

Orion	0.25 ± 0^{c}	0.27 ± 0.03^{c}	0.23 ± 0^{bc}	0.23 ± 0.01^{bc}	0.22 ± 0.02^{bc}	0.16 ± 0.04^{ab}	0.12 ± 0.01^{a}
Frontier	0.27 ± 0^{h}	0.22 ± 0^{def}	0.22 ± 0^{cdef}	0.22 ± 0^{cdef}	0.19 ± 0^{abc}	0.18 ± 0.01^{ab}	0.17 ± 0^{a}
Correlation with protein					**R = -0.19**		
Correlation with starch					**R = 0.28**		
			Emulsion Capacity (%)				
Crown	36.94 ± 1.9^{ab}	36.84 ± 0^{bcde}	33.33 ± 0^{a}	36.84 ± 0^{bcde}	39.44 ± 0.55^{de}	36.84 ± 0.8^{bcde}	40.00 ± 0^{e}
Royal	37.47 ± 0.8^{d}	36.84 ± 0^{abc}	36.84 ± 0^{abc}	33.33 ± 0^{a}	35.00 ± 0^{cd}	35.00 ± 0.1^{cd}	36.84 ± 0^{cd}
Sierra	36.84 ± 0^{ab}	42.5 ± 2.5^{c}	40.00 ± 0.01^{bc}	35.00 ± 0^{a}	40.00 ± 0^{bc}	40.00 ± 0^{bc}	36.84 ± 0^{ab}
Orion	35.92 ± 0.9	45.00 ± 0^{d}	36.84 ± 0^{abc}	35.00 ± 0^{ab}	40.00 ± 0^{c}	40.00 ± 0^{c}	35.00 ± 0^{ab}
Frontier	36.84 ± 0^{cd}	38.09 ± 0^{d}	28.57 ± 0^{a}	28.63 ± 1.8^{a}	38.09 ± 0^{d}	33.33 ± 0^{abcd}	30.95 ± 2.8^{ab}
Correlation with protein					**R = -0.36**		
Correlation with starch					**R = 0.31**		
			Foaming Capacity (%)				
Crown	220 ± 0^{c}	23.33 ± 3^{ab}	23.33 ± 10^{ab}	6.66 ± 0^{a}	33.33 ± 0^{ab}	66.66 ± 33^{ab}	133.33 ± 0^{bc}
Royal	$266.66 \pm 20e$	83.33 ± 50^{abc}	50.0 ± 16^{abc}	6.67 ± 0^{ab}	53.33 ± 20^{abc}	30.0 ± 20^{ab}	0^{a}
Sierra	333.33 ± 17^{c}	20 ± 13^{a}	110 ± 17^{ab}	60 ± 10^{ab}	83.33 ± 50^{ab}	26.67 ± 13^{a}	66.67 ± 13^{ab}
Orion	203.33 ± 27^{c}	80 ± 13^{a}	56.67 ± 23^{a}	66.67 ± 33^{a}	66.67 ± 33^{a}	100 ± 23^{ab}	76.67 ± 56^{a}
Frontier	190 ± 10^{c}	73.33 ± 40^{ab}	10 ± 3^{a}	10 ± 3^{a}	20 ± 10^{ab}	0^{a}	6.67 ± 0^{a}
Correlation with protein					**R = -0.05**		
Correlation with starch					**R = 0.37**		

LSD was performed for the "day," "temperature," and "RH" factors within three-way ANOVA for data. The LSD values are the same for all factors (day, temperature, and RH) because they are calculated based on the overall mean square error from the ANOVA, which is consistent across all factors (true replicate = 2 and total n = 4). Values with lowercase letters means no significant difference among treatments. Data points represent the mean ± standard deviation of two independent experiments. Different letters indicate statistically significant differences in varieties ($p \leq 0.05$).

2.3.6. Color differences

For color evaluation, L*, a*, b*, and ΔE values were observed to understand the effects of the high temperature and RH on chickpea seeds stored for 360 days (Figure 2.5 and Table 2.7). Color values were affected by variables significantly. L* value decreased for all samples, reflecting darkening after long-term high temperature and 65% RH storage conditions. These conditions can promote the occurrence of enzymatic browning, which is an enzymatic reaction of polyphenol oxidase that results in the polymerization of phenols and contributes to changes in color in the stored samples (Sikora & Świeca, 2018). Frontier had the lowest a* value, which increased from -1.01 at day 0 to -0.31 at day 360 of storage. Crown had the highest b* values (19.53 to 19.64) on day 0 and day 360, respectively. The b* value decreased for all samples meaning that yellowness decreased, which can be due to enzymatic reactions under high temperature and RH resulting in a brown color (Maninder et al., 2007). ΔE indicated a total color difference between zero-day samples and 180 and 360-day samples in the same condition (Table 2.7). Temperature, RH, and day had significant effects on ΔE (in 180- and 360-day sampling) in all varieties. Orion, Royal, and Crown had the highest ΔE at 360-day sampling with 40 ℃ and 65% RH conditions. Overall, the reduction in lightness and greenness values likely contributed to the higher ΔE values.

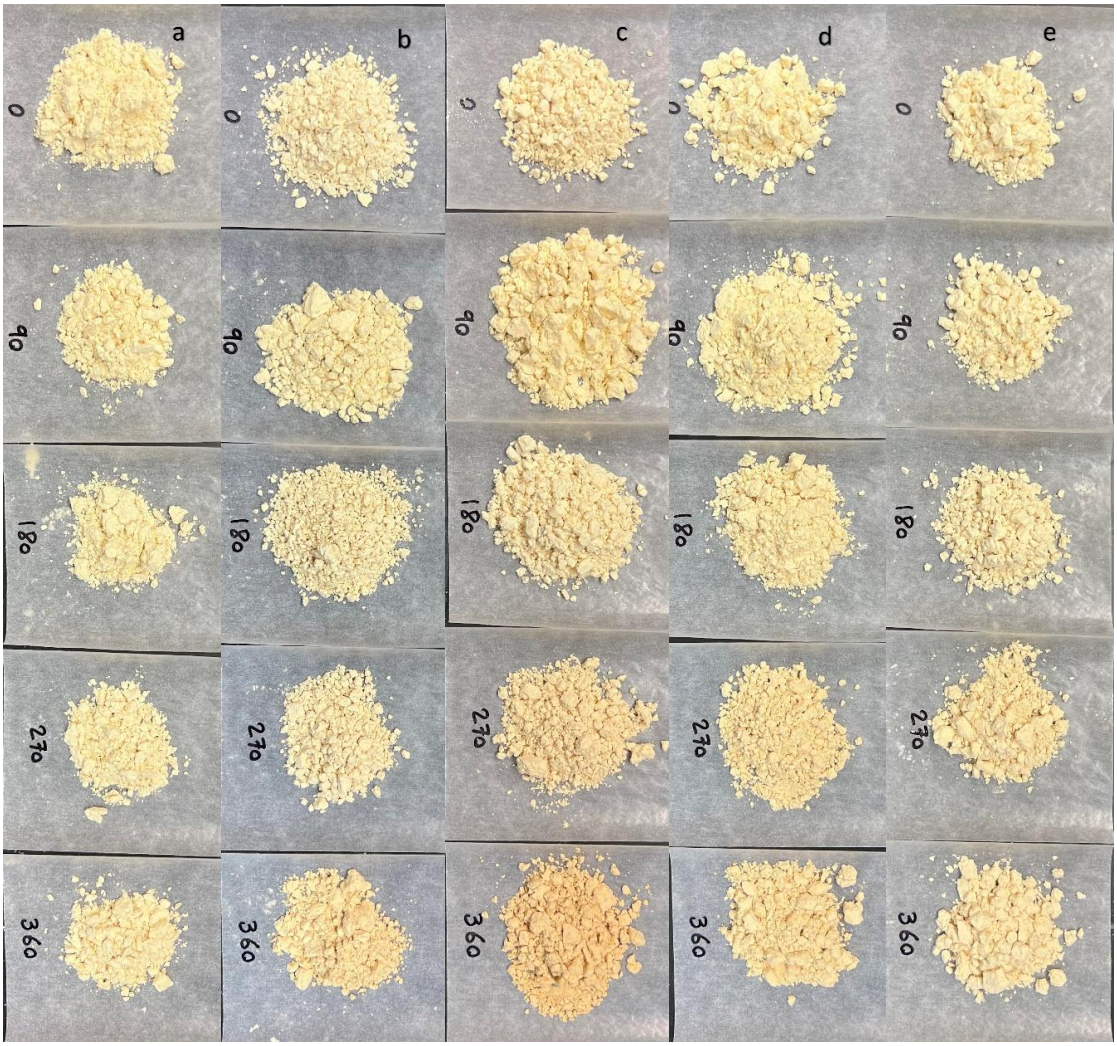

Figure 2.5. The color of flours obtained from different cultivars (Frontier (a), Orion (b), Royal (c), Crown (d), and Sierra (e)) of chickpea stored over multiple days (0, 90, 180, 270, and 360) under harsh condition (65% RH and 40 °C). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2.7. Effects of the variables (Day, RH, and Temperature) on color properties (L*, a*, b*, ΔE) of all chickpea varieties (Crown, Royal, Sierra, Orion, and Frontier).

Variety	Day-0	Day-180					
		21℃			40℃		
		40%	55%	65%	40%	55%	65%
				L*			
Crown	88.34 ± 0.1^{c}	88.25 ± 0.2^{bc}	88.37 ± 0.3^{bc}	88.15 ± 0.1^{bc}	87.55 ± 0.3^{bc}	87.32 ± 0.9^{bc}	87.16 ± 0.9^{bc}
Royal	88.27 ± 0^{b}	88.37 ± 0.3^{b}	88.42 ± 0.3^{b}	88.44 ± 0.3^{b}	88.33 ± 0.4^{b}	88.49 ± 0.1^{b}	86.95 ± 1.5^{ab}
Sierra	88.56 ± 0^{b}	88.99 ± 0.2^{b}	88.95 ± 0.1^{b}	88.31 ± 0^{b}	88.98 ± 0.1^{b}	88.20 ± 0.2^{b}	87.36 ± 0.5^{ab}
Orion	87.99 ± 0.1	88.23 ± 0.4	87.57 ± 0.8	86.72 ± 0.2	88.14 ± 0	86.53 ± 1.4	85.68 ± 0.9
Frontier	87.67 ± 0.2^{abc}	88.22 ± 0.1^{c}	87.69 ± 0.4^{abc}	87.80 ± 0.3^{abc}	87.93 ± 0.4^{bc}	86.41 ± 0.8^{abc}	87.24 ± 0^{abc}
				a*			
Crown	-0.71± 0.1^{a}	-0.75 ± 0.1^{a}	-0.81 ± 0^{a}	-0.74 ± 0^{a}	-0.35 ± 0.1^{ab}	-0.20 ± 0.3^{ab}	0.02 ± 0.4^{ab}
Royal	-0.85 ± 1.3^{a}	-0.64 ± 0.1^{a}	-0.65 ± 0.2^{a}	-0.75 ± 0^{a}	-0.43 ± 0.1^{a}	-0.55 ± 0^{a}	0.09 ± 1.1^{ab}
Sierra	-0.73 ± 1.5^{a}	-0.44 ± 0.1^{ab}	-0.53 ± 0.1^{a}	-0.53 ± 0.1^{a}	-0.36 ± 0.2^{ab}	-0.28 ± 0^{ab}	0.0 ± 0.3^{ab}
Orion	-0.71 ± 1.4^{a}	-0.56 ± 0.1^{a}	-0.55 ± 0.1^{a}	-0.69 ± 0.2^{a}	-0.39 ± 0.1^{a}	-0.09 ± 0.6^{a}	-0.05 ± 0.5^{a}
Frontier	-0.99 ± 0^{a}	-0.83 ± 0^{a}	-0.73 ± 0.1^{a}	-0.74 ± 0^{a}	-0.62 ± 0.1^{abc}	0.18 ± 0.2^{cd}	-0.27 ± 0ab^{cd}
				b*			
Crown	19.14 ± 0.1^{abc}	19.12 ± 0.2^{abc}	19.78 ± 0.4^{bcd}	19.88 ± 0.1^{bcd}	18.56 ± 0.3^{ab}	18.33 ± 0.3^{ab}	17.84 ± 0.5^{a}
Royal	19.33 ± 0	17.93 ± 0.6	18.5 ± 0.2	19.14 ± 0.7	17.06 ± 0.4	17.28 ± 0.4	18.62 ± 0.7
Sierra	17.26 ± 0^{cd}	15.62 ± 0.3^{abcd}	16.05 ± 0.2^{abcd}	16.76 ± 0.2^{abcd}	14.78 ± 0.3^{a}	15.25 ± 0.2^{abc}	16.49 ± 0.1^{abcd}
Orion	18.4 ± 0^{abc}	17.41 ± 0.4^{ab}	19.26 ± 0.2^{bcd}	19.23 ± 0.2^{bcd}	17.36 ± 0^{ab}	18.18 ± 0.1^{abc}	19.31 ± 0.1^{bcd}
Frontier	17.1 ± 0.1^{abc}	16.8 ± 0.1^{abc}	17.6 ± 0.1^{c}	17.6 ± 0.2^{c}	16.3 ± 0.4^{a}	17.5 ± 0.2^{bc}	17.5 ± 0.1^{bc}
				Δ*E*			
Crown	0^{a}	1.53 ± 0.1^{ab}	1.01 ± 0.1^{bcd}	0.95 ± 0.3^{bcd}	1.53 ± 0.1^{bcd}	2.04 ± 0.2^{cd}	2.66 ± 0.1^{d}
Royal	0^{a}	1.48 ± 1.1^{ab}	0.98 ± 0.4^{ab}	0.80 ± 0.2^{ab}	1.61 ± 0.6^{ab}	1.33 ± 0.6^{ab}	1.86 ± 1.1^{ab}
Sierra	0^{a}	1.72 ± 0.6^{ab}	1.29 ± 0.1^{ab}	0.60 ± 0.2^{ab}	1.65 ± 0.6^{ab}	1.19 ± 0.5^{ab}	1.30 ± 0.4^{ab}
Orion	0^{a}	1.02 ± 0.8^{ab}	1.18 ± 0.6^{ab}	1.56 ± 0.3^{ab}	2.75 ± 0.1^{ab}	2.56 ± 0.3^{ab}	1.68 ± 1.1^{ab}
Frontier	0^{a}	0.70 ± 0.3^{abc}	0.58 ± 0.2^{abc}	0.87 ± 0.2^{abc}	0.78 ± 0.1^{abc}	2.10 ± 0.8^{bc}	1.31 ± 0.1^{abc}

Variety	Day-0	Day-360					
		21℃			40℃		
		40%	55%	65%	40%	55%	65%
				L*			
Crown	88.34 ± 0.1^{c}	87.79 ± 0^{bc}	87.51 ± 0.3^{bc}	87.31 ± 0.1^{bc}	87.74 ± 0.0^{bc}	86.23 ± 0.7^{b}	81.86 ± 0.1^{a}
Royal	88.27 ± 0^{b}	86.96 ± 0.1^{b}	88.37 ± 0^{b}	88.07 ± 0.3^{b}	88.16 ± 0^{b}	87.07 ± 0.3^{ab}	81.89 ± 3.8^{a}
Sierra	88.56 ± 0^{b}	88.31 ± 0.1^{b}	87.61 ± 0.7^{ab}	88.21 ± 0.2^{b}	88.23 ± 0.5^{b}	84.52 ± 2.1^{a}	85.73 ± 1.3^{ab}
Orion	87.99 ± 0.1	87.97 ± 0.2	87.42 ± 0	87.15 ± 0	87.80 ± 0.2	83.78 ± 3.5	80.11 ± 0
Frontier	87.67 ± 0.2^{abc}	87.42 ± 0.1^{abc}	87.92 ± 0.1^{bc}	87.22 ± 0.2^{abc}	87.04 ± 0.1^{abc}	85.97 ± 0.7^{a}	86.18 ± 0.6^{ab}
				a*			
Crown	-0.71± 0.1^{a}	-0.60 ± 0.1^{ab}	-0.60 ± 0^{ab}	-0.56 ± 0^{ab}	-0.19 ± 0.1^{ab}	0.25 ± 0.3^{b}	2.36 ± 0.2^{c}
Royal	-0.85 ± 1.3^{a}	-0.39 ± 0.2^{a}	-0.59 ± 0^{a}	-0.68 ± 0^{a}	-0.17 ± 0^{a}	-0.08 ± 0^{a}	2.52 ± 3.0^{b}
Sierra	-0.73 ± 1.5^{a}	-0.44 ± 0^{ab}	-0.60 ± 0.1^{a}	-0.57± 0^{a}	-0.26 ± 0.1^{ab}	1.09 ± 1.0^{b}	0.73 ± 0.8^{ab}
Orion	-0.71 ± 1.4^{a}	-0.56 ± 1.1^{a}	-0.73 ± 0.1^{a}	-0.65 ± 0.7^{a}	0.11 ± 0.2^{a}	1.09 ± 1.3^{a}	3.56 ± 0.1^{b}
Frontier	-0.99 ± 0^{a}	-0.66 ± 0^{ab}	-0.74 ± 0^{a}	-0.64 ± 0^{ab}	-0.28 ± 0.2^{abcd}	0.31 ± 0.3^{d}	0.21 ± 0.2^{bcd}
				b*			
Crown	19.14 ± 0.1^{abc}	19.72 ± 0^{bcd}	20.64 ± 0.1^{cd}	19.9 ± 0.1^{bcd}	17.85 ± 0.2^{a}	18.89 ± 0.2^{ab}	19.87 ± 0.1^{bcd}
Royal	19.33 ± 0	18.68 ± 0.5	18.27 ± 0.2	19.17 ± 1.2	16.72 ± 0.1	17.48 ± 0.6	19.87 ± 0.6
Sierra	17.26 ± 0^{cd}	15.73 ± 0.1^{abcd}	16.17 ± 0.3^{abcd}	16.99 ± 0.2^{bcd}	15.04 ± 0.2^{ab}	17.46 ± 0.5^{d}	16.67 ± 0.2^{abcd}
Orion	18.4 ± 0^{abc}	17.97 ± 0.2^{abc}	17.94 ± 0.4^{abc}	19.98 ± 0.2^{cd}	16.99 ± 0.1^{a}	18.39 ± 0.4^{abc}	20.87 ± 0^{d}
Frontier	17.1 ± 0.1^{abc}	16.5 ± 0^{ab}	16.9 ± 0^{abc}	17.9 ± 0.1^{c}	16.8 ± 0.2^{abc}	17.4 ± 0.2^{bc}	17.3 ± 0^{abc}
				ΔE			
Crown	0^{a}	1.02 ± 0.1^{abc}	1.85 ± 0.5^{bcd}	2.18 ± 0.4^{cd}	2.17 ± 0.3^{cd}	2.40 ± 0.5^{d}	6.88 ± 0.1^{e}
Royal	0^{a}	1.11 ± 0.6^{ab}	1.10 ± 0.4^{ab}	1.45 ± 0.1^{ab}	1.83 ± 0.2^{ab}	1.78 ± 0.5^{ab}	6.99 ± 0.2^{b}
Sierra	0^{a}	1.58 ± 0.1^{ab}	1.48 ± 0.9^{ab}	0.54 ± 0.0^{ab}	1.46 ± 0.4^{ab}	4.47 ± 0.3^{b}	3.08 ± 1.2^{ab}
Orion	0^{a}	0.56 ± 0.3^{ab}	0.98 ± 0.4^{ab}	1.85 ± 0.3^{ab}	3.02 ± 0.4^{ab}	5.03 ± 0.7^{bc}	8.26 ± 0.1^{c}
Frontier	0^{a}	0.74 ± 0.0^{abc}	0.45 ± 0.2^{ab}	0.94 ± 0.1^{abc}	1.16 ± 0.4^{abc}	2.46 ± 0.9^{c}	2.18 ± 0.6^{bc}

The LSD values are the same for all factors (day, temperature, and RH) because they are calculated based on the overall mean square error from the ANOVA, which is consistent across all factors (true replicate = 2 and total n = 4). Values with lowercase letters means no significant difference among treatments. Data points represent the mean ± standard deviation of two independent experiments. Different letters indicate statistically significant differences in varieties ($p \leq 0.05$)

2.4. Conclusion

The investigation of 5 different chickpea cultivars under different storage conditions (40, 55, and 65% RH, and 21 and 40 °C) revealed that temperature and relative humidity (RH) significantly impacted chickpea functional properties, and color while it has no significant effects on chemical composition specifically protein, starch and fat. This means when there is no change in protein and starch, major changes in functional properties such as foaming, emulsion and pasting properties are because of the structural changes. Furthermore, protein and starch structural features were impacted by harsh (40 °C, 65% RH) storage conditions. Moisture content increased over time, with higher temperatures affecting moisture retention differently among cultivars. Functional properties such as water-holding capacity, oil-holding capacity, and pasting properties were affected by storage conditions, with notable differences among cultivars. For example, Frontier with high content of protein compared to other varieties had significant increase of WHC after 180 days of storage but after 360 days of storage under harsh condition, WHC decreased. This means 180 days for Frontier is considerable time because functional properties were improved. Also, Frontier had higher significant OHC compared to other samples after 360 days under harsh condition (65% and 40 °C). the foaming and emulsion properties decreased for all samples and varieties after 360 days for all conditions. The analysis of protein through SDS-PAGE and FT-IR revealed changes in molecular weight distribution, secondary protein structure, and new interactions. For examples some important bands in SDS-PAGE analysis disappeared that can be result of aggregation and insoluble structure of protein formation under harsh condition after 360 days of storage. Starch analysis via SEM showed protein-coated starch granules after storage, suggesting protein-starch

interactions. Amylose and amylopectin ratios changed, indicating alterations in starch composition. Pasting viscosities and gel firmness increased, while foaming capacity decreased during storage. Color analysis showed darkening and changes in color parameters, with variations among cultivars. However, it is noteworthy that extending the harsh storage conditions beyond 180 days might not be advisable, as many undesirable changes in functional properties were observed to initiate around this time point. Thus, RH and temperature in storage management are essential to maintain the quality and functionality of chickpea flour over prolonged periods. Storage at high temperature (41 °C) and relative humidity above 55% should be avoided as significant changes occurred in chickpea composition and functionality under these storage conditions. These results suggested that when seeds are stored in harsh condition over 180 days, protein isolation might be difficult due to the structural changes and aggregation formation.

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Chapter three

Water-soluble vitamin profile of different varieties of chickpea stored for 360 days under high temperatures and high relative humidity

This chapter was prepared to be published. It is under prapreation process.

3. Abstract

Legumes, including chickpeas are recognized as valuable sources of plant-based food, rich in nutrients such as protein, carbohydrates, minerals, and B-group vitamins. Using high-performance liquid chromatography (HPLC) equipped with a UV detector, the impact of storage conditions (40°C and 65% relative humidity) for 360 days on the vitamin B profile of chickpea varieties, including Crown, Royal, Orion, Sierra, and Frontier were determined, and significant differences were observed ($P \leq 0.05$).

Thiamin, riboflavin, niacin, pyridoxine, and folic acid were analyzed. Validation of the measuring method was conducted, ensuring sensitivity, precision, and linear range for each vitamin compound. Correlation coefficient confirmed the accuracy of the standard curves ($R^2 > 0.99$). The limit of detection (0.22-0.26 μg/mL) and the limit of the quantification (0.43-0.52 μg/mL) and matrix effects (82-103%) were evaluated for chickpea blank samples. Results supported significant ($P \leq 0.05$) decreases in vitamin B content across all chickpea varieties over the storage period. Vitamins changed by the effects of temperature (T), day (D), relative humidity (RH), and the effects of the interaction of the variables.

Thiamin (34-75 μg/100g), riboflavin (47-117 μg/100g), niacin (211-590 μg/100g), pyridoxine (463-743 μg/100g), and folic acid (81-293 μg/100g) concentrations varied among the varieties and storage conditions. Notably, the interaction of temperature, relative humidity, and storage duration influenced (4-80% reduction) the vitamin B profile of chickpeas. Riboflavin content had the most significant ($P \leq 0.05$) reduction (79%), followed by folic acid (69%), thiamin (55%), niacin (48%), and pyridoxin (15%). To

ensure the preservation of chickpea seed quality for over a year, it is imperative to control temperature and humidity levels during storage.

Keywords: water-soluble vitamin, legume, storage, HPLC, chickpea

3.1. Introduction

Legumes are a valuable source of plant-based food around the world. Legume consumption is a growing trend in developed countries and most grown legumes are soybeans, peanuts, beans, peas, chickpeas, and lentils (Prodanov et al., 2004). Plant-based food alternatives sales increased by 8% annually from 2010 to 2017 and legumes gained global interest as a source of protein. Legumes consumption leads to a decrease in the risk of cardiovascular disease, hypertension, obesity, type 2 diabetes, and dyslipidemia (Andac-Ozturk et al., 2022). Legumes are rich in nutrients such as protein, carbohydrates, minerals, and B-group vitamins (Siitonen et al., 2024).

The B vitamins are water-soluble components that have a vital metabolic role in the conversion of carbohydrates to energy and the biosynthesis of amino acids, fatty acids, and nucleotides in an organism (Kennedy, 2016; Witten & Aulrich, 2018). The B vitamins has a crucial role in human health including immune regulation and prevention of beriberi. Furthermore, B vitamins are important in controlling heart failure, type 2 diabetes, cancer, and cardiometabolic diseases (Yang et al., 2024). All the B vitamins except $B_{12,}$ are produced in plants and are obtainable from plant-based foods. In food and biological tissue, vitamin B_1 is found in the form of thiamine pyrophosphate and thiamine monophosphate, and B_2 is found in the form of flavin mononucleotide and flavin adenine dinucleotide (Akça et al., 2019). Also, niacin is in the form of nicotinamide dinucleotide and nicotinamide dinucleotide phosphate in plant tissue and these coenzymes act as key factors for pyruvate dehydrogenase enzyme in the citric acid cycle (Demir et al., 2023).

Legumes contain different types of B vitamins such as thiamin (B_1), riboflavin (B_2), niacin (B_3), pyridoxine (B_6), and folic acid (B_9) (Marshall et al., 2021). Legumes, including

chickpeas, have a broad spectrum of vitamin B content. For instance, the reported ranges for various B vitamins per 100 grams in chickpeas are as follows: 320-700 µg for thiamin, 30-540 µg for riboflavin, 220-530 µg for pyridoxine, 1590-1615 µg for niacin, and 420-537 µg for total folic acid (Marshall et al., 2024; Sehar et al., 2023). In a different report, vitamin B content was 173 µg/100g for riboflavin, 453 µg/100g for thiamin, and 466 µg/100g for pyridoxine (Alajaji & El-Adawy, 2006).

Legumes are cultivated, harvested, and stored in different relative humidity (RH) and a wide range of temperatures (T) 5-40°C. High T and RH are key factors and lead to changes in chemical, functional, and nutritional properties (Malhotra et al., 2023). According to our previous research (Kazemzadeh pournaki et al., 2024), the combination of high temperature (40°C) and relative humidity (65%) significantly altered the functional properties and chemical composition of protein and starch isolated from various chickpea varieties after 360 days of storage. Additionally, it is imperative to assess and evaluate the impact of these adverse conditions on the vitamin B profile of legumes, as it constitutes another crucial aspect of their nutritional composition.

Currently, one of the approved techniques for analyzing vitamin B is high-performance liquid chromatography (HPLC) equipped with a UV detector. This method enables the precise quantification of the vitamin B complex in biological samples with exceptional specificity and sensitivity (Sasaki et al., 2020). There is no information in the literature about the impacts of storage on B vitamin of chickpea. Thus, the objective of this study was to determine the effects of storage temperature and relative humidities (21 °C and 40%, 40 °C and 40%, 21°C and 65%, and 40 °C and 65%) and days on the vitamin B profile of chickpea. The hypothesis of this research was that the storage conditions that

had the greatest impact on the functionality of chickpea flour also had the greatest impact on B vitamin concentrations.

3.2. Material and Methods

3.2.1. Chemicals

Five distinct B vitamin standards were procured from TCI Chemicals (Tokyo, Japan), each with purity exceeding 98% HPLC grade. These standards include thiamine hydrochloride, riboflavin tetrabutyrate, niacin (nicotinic acid), pyridoxine hydrochloride, and folic acid (Pteroyl-L-glutamic acid), acetonitrile, β-mercaptoethanol, aluminum acetate, acetic acid, HPLC water, porcine trypsin, porcine protease, rat serum, phosphate monosodium, and phosphate disodium were procured from Sigma-Aldrich. All solutions containing enzymes and standards were prepared using HPLC-grade water. Mobile phases and buffer solutions were accurately filtered through a PTFE membrane filter (0.45 µm pore, and 25 mm diameter) (Sigma-Aldrich).

3.2.2. Experimental design

Samples included the chickpea varieties Crown, Royal, Sierra, Orion, and Frontier. Chickpea storage conditions (temperature and relative humidity (RH)) was specified as LTLRH (21 ℃ and 40%), HTLRH (40 ℃ and 40%), LTHRH (21℃ and 65%), and HTHRH (40 ℃ and 65%). The experimental design of this project was factorial design (2×2) with repeated measurement at zero and 360 days of the storage. Samples were stored 360 days in sealed container with a hygrometer and humidity bag (65%, 1 bag/100 g seed)

to control temperature and RH. To increase temperature, samples were stored in an oven at 40 ± 0.5 ℃. After sample collection, they were stored in dark-brown color bottles, then samples were milled and stored in a -40 ± 0.5 ℃ freezer until the B vitamin extraction.

3.2.3. B_1, B_2, B_3, B_6, and B_9 vitamins quad-enzyme extraction

To achieve optimal conditions for B vitamin recovery from chickpea flour, the pH of the buffer was adjusted to 5.5, and quad-enzyme (α-amylase, protease, porcine trypsin, and rat serum) was utilized for extracting B vitamins (Figure 3.1). To prevent photooxidation, the sample extraction process was conducted in subdued light and the temperature (21℃ ± 0.5) of the room was controlled. The extraction protocol was a modified method of Agyenim-Boateng et al. (2022). Milled chickpea seeds (50 mg) were placed into a 1.5 mL screw-cap tube, followed by mixing with 1 mL of 50 mM phosphate buffer (sodium phosphate pH 5.5) containing 2% β-mercaptoethanol. The mixture was heated in a boiling water bath for 10 minutes and was then cooled on ice for an additional 10 minutes. Then, 20 µL of α-amylase was added, and the sample solution was incubated at 37 °C with shaking for 30 min. After incubation, the sample was boiled for 5 minutes to inactivate the enzymes, followed by cooling on ice for another 10 minutes. Following, 30 µL of protease was added, and the sample was incubated for 1 h at 37 °C. The sample was once again boiled for 5 minutes to deactivate the enzymes. The porcine trypsin (30 µL) was added, and the solution was incubated for 1 h and once again boiled for 5 minutes to deactivate the enzymes. The final step was to add 50 µL rat serum, for deconjugation of polyglutamylated group of folates, to the solution that was then incubated for 4 hours at 37 °C. Subsequent steps involved boiling the sample for 10 minutes, cooling on ice for 10

minutes, and centrifugation (Thermo Scientific™ Sorvall™ Legend™ Micro 17 Microcentrifuge) twice at 17000 × g for 10 minutes at 4 °C. Finally, 100 µL of the filtrate was taken for analysis, and the remaining sample was stored at −80 °C.

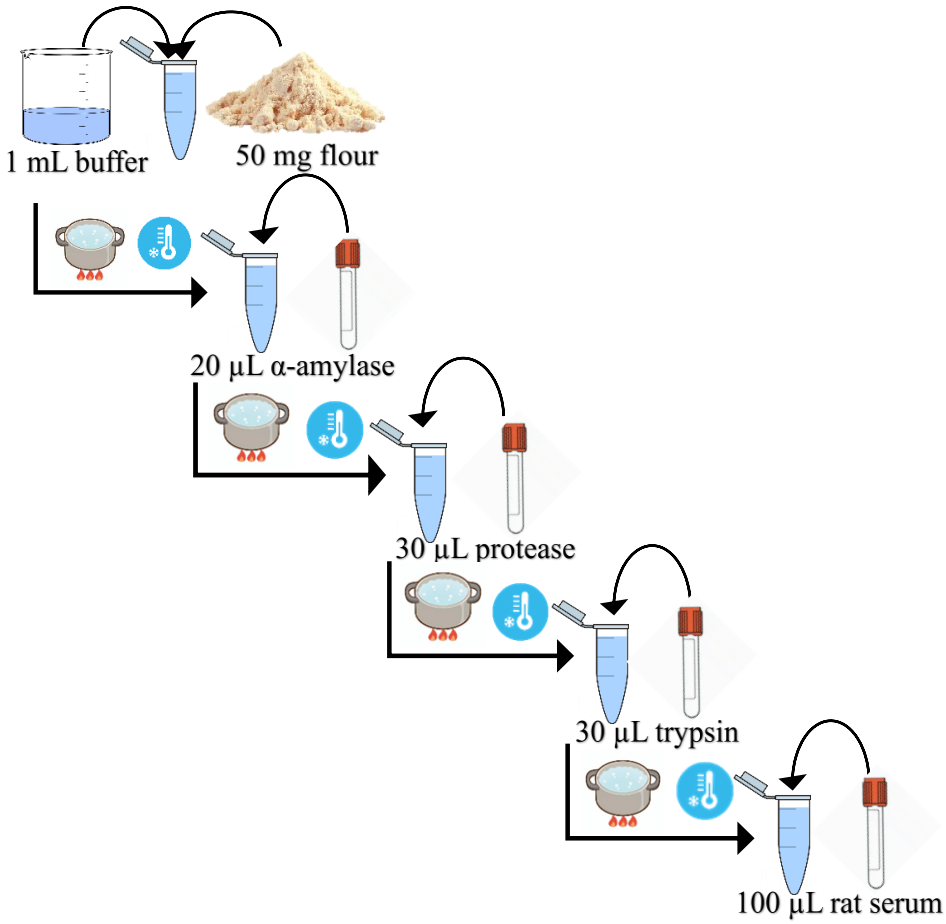

Figure 3.1. B_1, B_2, B_3, B_6, and B_9 vitamins quad-enzyme extraction steps for chickpea flour. Each step of heating was 5 min at 95 °C and cooling for 10 min in ice bath. The final volume of the samples with buffers and enzymes was 1.5 mL.

3.2.4. Validation of measuring method

Blank samples were precisely prepared to employ a chickpea matrix as described by Agyenim-Boateng et al. (2022). The fine-milled flours of chickpea sieved through a 0.05 mm screen, were blended with 50 mM phosphate buffer at a pH of 5.5, each at a 50

mg/mL concentration. The phosphate buffer utilized for the blank matrix was deliberately devoid of any antioxidants. Subsequently, the flour solutions were subjected to a boiling process at 100 °C for 1 hour, concurrently exposed to direct sunlight to induce the degradation of endogenous water-soluble vitamins. Following this, the samples underwent centrifugation at 16,000 × g for 30 min, and 10% active charcoal was introduced to the supernatant. The resultant mixture was vigorously shaken for 1 h and then centrifuged at 16,000 × g for 30 minutes. The supernatant was then filtered using a 0.45 µm PTFE pre-slit septa filter vial (Agilent, USA). The filtered supernatant was used to determine the absence of B vitamins within the detection limit. All samples were stored at -80 °C until needed for the analysis on the HPLC.

For method validation, sensitivity, matrix effects, the parameters of the limit of detection (LOD), the limit of quantification (LOQ), slope, correlation coefficient, and linear range were determined (Matuszewski et al., 2003). This determination was facilitated using a 6–8-point calibration curve with a blank matrix (n=5). The methodology thus ensured a comprehensive evaluation of the analytical performance and reliability of the developed procedure. The limit of detection (LOD), and limit of quantification (LOQ) were determined based on the signal-to-noise (S/N) ratio by 3 and 10, respectively. The standard samples were spiked to both blank sample and standard buffer to analyze the effects of the matrix on vitamin detection. The difference of standard vitamin concentration between matrix and buffer $(C_B\text{-}C_S)\times100/C_B$ (C_B is blank spiked vitamin concentration and C_S is standard in buffer solution) were calculated and expressed as percentage (Matuszewski et al., 2003). Recovery of the vitamins were evaluated to prove extraction method accuracy and effect on vitamin final concentration. Recovery was calculated by

addition of the standard vitamin solutions to flour and blank samples and qual-enzyme extraction method explained above applied to evaluate vitamin final concentration.

3.2.5. Stock solutions and vitamin standards

All vitamins (thiamin (B_1), riboflavin (B_2), niacin (B_3), pyridoxine (B_6), and folic acid (B_9)) were prepared at 100 µg/mL under subdued light to prohibit photooxidation using sodium phosphate buffer 50 mM at pH 5.5 containing 2% β-mercaptoethanol (Riaz et al., 2019). The concentration of calibration solutions was prepared from 0.02 µg/mL to 100 µg/ml).

3.2.6. Determination of B vitamins by HPLC

Samples were analyzed using a 1260 Infinity Agilent HPLC (Waldbronn, Germany) equipped with a binary pump (1260 Quat Pump, G7111B), autosampler (1260 Vial sampler, G7129A), infinity diode-array detector (1260 DAD WR, G7115A), and column heater. All 5 water-soluble vitamins were separated on a Poroshell 120 HILIC-OH5 column (inner diameter 2.1 mm, particle size 2.7 µm, pore size 120 Å, and length 150 mm, Agilent, USA). Column temperature of 40 ± 0.4°C was used for the complete separation of all vitamins. Sample injection volume was 1.0 µL. The mobile phase was water with 100 mM ammonium acetate and 0.5% acetic acid (a) and acetonitrile (b). The mobile phase composition and gradient (Table 2.1) were 87% B for 0.5 min, 87-50% B in 6 min, and 3 min re-equilibration to 13% A and 87% B. The detection wavelength of components and frequency of detection was 260 nm and 80 Hz using a wide-range diode array detector. Results and peaks were analyzed with a CQL data analyzer by Agilent.

Table 3.1. Gradient program of mobile phases for vitamin B profiling in HPLC

Time (min)	Mobile phase A *(%)	Mobile phase B **(%)	Flow rate (mL/min)
0.0	13	87	0.5
0.5	87	50	0.5
6.0	50	50	0.5
9.0	13	87	0.5

*A was 100 mM ammonium acetate and 0.5% acetic acid.
**B was acetonitrile.

3.2.7. Statistical analysis

There are 4 combinations of storage RH and T factors, all varieties were stored in storage conditions in 2 replications and extraction was applied in 2 replications for each sample (N = 4). The mean vitamin value was used in the comparison for statistical differences. These were 1) 40% and 21 ℃, 2) 40% and 40 ℃, 3) 65% and 21 ℃, 4) 65% and 40 ℃. Two days of sample collection were chosen including zero-day and 360-day to compare vitamin content changes from 0 to 360 days of storage under harsh and controlled conditions. Results were analyzed by analysis of the variance (two-way ANOVA) with RStudio at a 95% confidence level. Time, cultivars, humidity, and temperature were the independent variables that affect dependent variables (B vitamins) and their interaction.

Precision was confirmed based on the relative standard deviation (RSD) of the peak area and retention time on the same day and different days of injection.

3.3. Results and Discussion

3.3.1. Validation

The limit of detection (LOD), the limit of quantification (LOQ), the linear range (LR), the slope of the equation, and the correlation coefficient (R^2) were evaluated for each vitamin compound (Table 3.2). Calibration curves were calculated using a blank chickpea matrix and n= 8 points were used to evaluate R^2 and the slope of the equation. Prepared standards were diluted and injected into the column to calculate calibration curves and dilution was continued to reach LOD and LOQ for each standard.

Table 3.2. Calibration and standardization data for B vitamin standards were prepared in a blank chickpea matrix.

Compounds	R^2	LOD* (µg. mL $^{-1}$)	LOQ* (µg. mL $^{-1}$)	LR* (µg. mL $^{-1}$)	Regression equation	Matrix effects %	Recovery (%)
Thiamin (B_1)	0.9917	0.22	0.44	0.44-100[C]	$y^a = 6.3511x^b - 2.9227$	90.89	96
Riboflavin (B_2)	0.9956	0.22	0.43	0.43-100	$y = 2.4526x + 0.4243$	102.93	100
Niacin (B_3)	0.9914	0.26	0.52	0.52-100	$y = 1.9684x - 3.6462$	87.83	85
Pyridoxine (B_6)	0.9952	0.22	0.43	0.43-100	$y = 0.717x - 0.2838$	82.55	70
Folic acid (B_9)	0.9981	0.26	0.51	0.51-100	$y = 2.4615x - 8.9842$	88.09	83

[a] y = mass concentration (µg. mL $^{-1}$)

[b] x = HPLC peak area

[C] means the highest standard concentration was 100 µg. mL $^{-1}$.

*LOD means Limit of Detection, LOQ means Limit of Quantification, and LR means Linear Range of Standards (n = 8).

The correlation coefficient ($R^2 > 0.99$) for standard curves (Table 3.2) supports a linearity of the calibration curves. Standard elution and retention times are indicated in Figure 3.2. The limits of detection (LOD) were determined as follows: 0.22 µg/100g for thiamin, 0.22 µg/100g for riboflavin, 0.26 µg/100g for niacin, 0.22 µg/100g for pyridoxine, and 0.26 µg/100g for folic acid. Additionally, the limits of quantification (LOQ) were determined as 0.44 µg/100g for thiamin, 0.43 µg/100g for riboflavin, 0.52 µg/100g for niacin, 0.43 µg/100g for pyridoxine, and 0.51 µg/100g for folic acid. Standard curves were arranged, and regression equations were calculated to determine the linear range of the curves, resulting in the following ranges: 0.44-100 µg/100g for thiamin, 0.43-100 µg/100g for riboflavin, 0.52-100 µg/100g for niacin, 0.43-100 µg/100g for pyridoxine, and 0.51-100 µg/100g for folic acid. All measured concentrations fell within these linear ranges, indicating the reliability of the analysis.

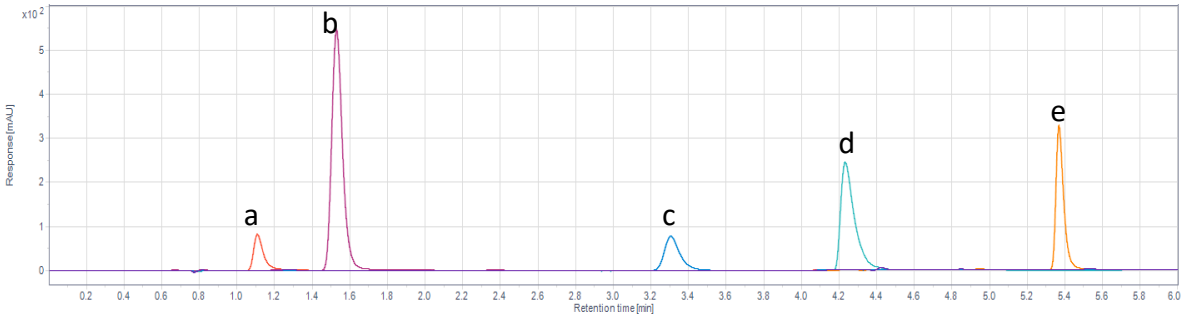

Figure 3.2. The retention time (min) of five vitamers along 6 minutes of separation, thiamin (a), riboflavin (b), niacin (c), pyridoxine (d), and folic acid (e).

To assess matrix effects, standard solutions (200 µg/mL) of all vitamins were added to both a blank chickpea matrix and buffer and detected peaks were compared (n=3). The observed matrix effects ranged from 82.5% to 102.9%. The matrix effect is in the range of the previous study of Agyenim-Boateng et al. (2022) who reported the matrix effect of

soybean in the folic acid ranged from 81 to 105%. The retention times (Figure 3.2) were 4.3 min for thiamin, 1.6 min for riboflavin, 3.3 min for niacin, 1.2 min for pyridoxin, and 5.4 min for folic acid. To evaluate the accuracy of the extraction procedures, recovery of standard vitamins was calculated with the spiked standard vitamin in actual samples and extraction was applied. All vitamin recovery was above 70%. For example, thiamin recovery after quad-enzyme extraction was 96% while riboflavin was recovered 100%. Also, 85%, 70% and 83% of niacin, pyridoxine, and folic acids were recovered after extraction. Agyenim-Boateng et al. (2022) calculated same recoveries for different vitamers of folates.

The intra-day and inter-day precision (expressed as RSD %) of all vitamin B peak areas and retention times fell within satisfactory ranges and presented run-to-run precision and robustness of the method (Table 3.3). Specifically, the RSD% range for peak areas was 1.65-6.41% for intra-day and 1.69-8.66% for inter-day analyses. Additionally, the RSD% range for retention times was 0.05-4.11% for intra-day and 0.25-3.17% for inter-day precision. These results indicate the reliability and consistency of the analytical method employed (<10%).

Table 3.3. Results from the evaluation of the intra- and inter-day precision of the LC method

Vitamin	Intra[a]-day area precision % RSD	Inter[b]-day area precision % RSD	Intra-day RT[c] precision % RSD	Inter-day RT precision % RSD
Thiamin ($B_{1)}$	6.41	8.66	0.05	1.11
Riboflavin ($B_{2)}$	1.65	1.69	4.11	3.17
Niacin (B_3)	5.48	2.07	0.91	2.35
Pyridoxine (B_6)	5.61	4.47	1.97	0.73
Folic acid (B_9)	2.14	3.12	0.09	0.25

[a]Intra-day n= 3
[b]Inter-day n= 3
[c]Retention time

3.3.2. Effects of the variables on the vitamin B profile of chickpea

Chickpea B vitamin profiles have been extensively studied, yet there remains limited information regarding the variations among different chickpea varieties. Among the essential vitamins analyzed, concentrations of thiamin, riboflavin, niacin, pyridoxin, and folic acid in chickpea were reported at 70-1500 µg/100g, 10-1700 µg/100g, 20,000 µg/100g, 45-2000 µg/100g, and 400 µg/100g, respectively (Andac-Ozturk et al., 2022; Sehar et al., 2023; Wallace et al., 2016). The Zero-day results (Figure 3.3) confirm that thiamin content in different varieties varied from 50.1 µg/100 g in Sierra to 75.0 µg/100g in Crown. The riboflavin content ranged from 93.1 µg/100g in Crown to 117.4 µg/100g in Sierra, while the niacin content varied from 420.8 µg/100g in Royal to 590.6 µg/100g in Crown. Additionally, Pyridoxin content ranged from 577.6 µg/100g in Frontier to 743.7 µg/100g in Orion, and Folic Acid content varied from 224.4 µg/100g in Frontier to 293.3 µg/100g in Crown. Thiamin, riboflavin, and pyridoxin are in the reported range for all chickpea varieties. According to USA Pulses technical manual (Anonymous, 2016), the thiamin content was 485 µg/100g, riboflavin was 106 µg/100g, niacin was 1762 µg/100g, pyridoxin was 492 µg/100g, and total folate was 437 µg/100g. Notably, the riboflavin and pyridoxin content observed in this project align with the range reported for pulses in the USA. All vitamins decreased over time of 360 days under various conditions (Figure 3.3), so it is important to compare 0-day samples with HTLRH, LTHRH, and HTHRH treatments. Sierra and Frontier had the highest accumulative vitamin B profile compared to Royal, that had the lowest accumulative vitamin B profile in HTHRH.

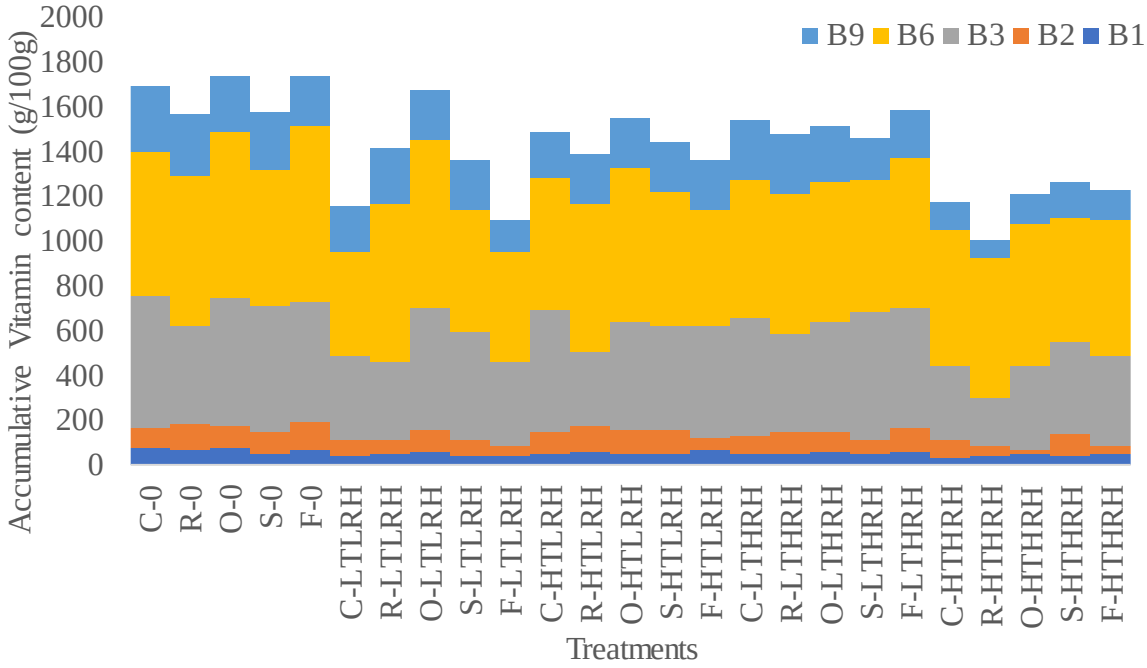

Figure 3.3. Treatments (0, LTLRH, HTLRH, LTHRH, and HTHRH) accumulative vitamin B profile (B_1, B_2, B_3, B_6, and B_9) concentration in different varieties (Crown (C), Royal (R), Orion (O), Sierra (S), and Frontier (F)).

The Vitamin B profile exhibited notable changes across all chickpea varieties (Table 3.4). Specifically, thiamin and riboflavin levels varied under different storage conditions, with a discernible decrease observed after 360 days of storage under 65% RH and 40°C (Figures 3.3 and 3.4).

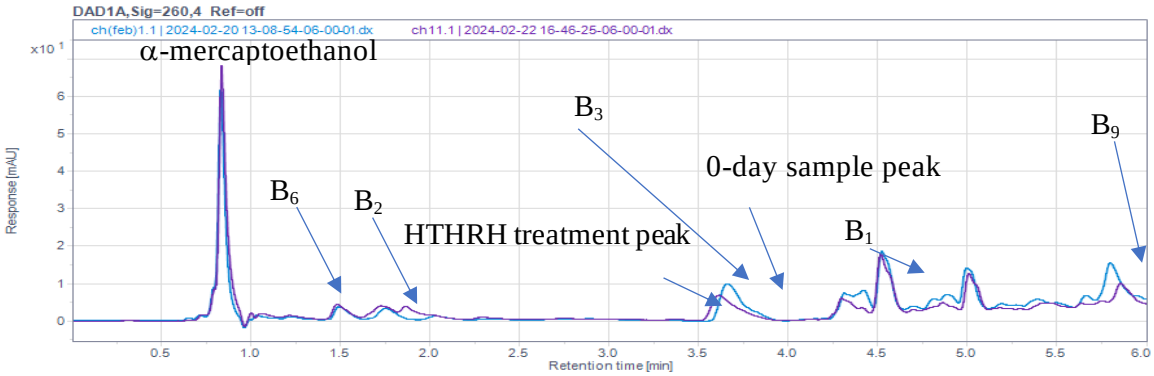

Figure 3.4. Crown vitamin B peaks (B_1, B_2, B_3, B_6, and B_9) at 0-day sample (blue) and HTHRH (purple).

Table 3.4. Effects of the variables and their interaction on different vitamins B (B_1, B_2, B_3, B_6, and B_9)

Variety	Vitamin	D[a]	T[a]	RH[a]	D×T×RH
Crown	Thiamin (B_1)	0.05>*	0.05>*	0.01>*	0.05>*
	Riboflavin (B_2)	0.05>*	0.15	0.13	0.05>*
	Niacin (B_3)	0.05>*	0.64	0.12	0.05>*
	Pyridoxine (B_6)	0.05>*	0.56	0.34	0.05>*
	Folic acid (B_9)	0.05>*	0.05>*	0.16	0.05>*
Royal	Thiamin (B_1)	0.99	0.05	0.05>*	0.05>*
	Riboflavin (B_2)	0.05>*	0.82	0.05>*	0.05>*
	Niacin (B_3)	0.05>*	0.05>*	0.89	0.05>*
	Pyridoxine (B_6)	0.05>*	0.30	0.05>*	0.05>*
	Folic acid (B_9)	0.05>*	0.05>*	0.05>*	0.05>*
Orion	Thiamin (B_1)	0.14	0.05>*	0.05>*	0.05>*
	Riboflavin (B_2)	0.05>*	0.05>*	0.05>*	0.05>*
	Niacin (B_3)	0.05>*	0.05>*	0.05>*	0.10
	Pyridoxine (B_6)	0.05>*	0.24	0.75	0.97
	Folic acid (B_9)	0.05>*	0.05>*	0.05>*	0.05>*
Sierra	Thiamin (B_1)	0.05>*	0.90	0.43	0.05>*
	Riboflavin (B_2)	0.05>*	0.05>*	0.20	0.59
	Niacin (B_3)	0.11	0.05>*	0.73	0.05>*
	Pyridoxine (B_6)	0.32	0.09	0.05>*	0.05>*
	Folic acid (B_9)	0.05>*	0.37	0.05>*	0.24
Frontier	Thiamin (B_1)	0.05>*	0.05>*	0.05>*	0.05>*
	Riboflavin (B_2)	0.05>*	0.05>*	0.05>*	0.05>*
	Niacin (B_3)	0.05>*	0.41	0.05>*	0.05>*
	Pyridoxine (B_6)	0.05>*	0.05>*	0.80	0.05>*
	Folic acid (B_9)	0.05>*	0.65	0.53	0.05>*

[a]Variables include day (D), relative humidity (RH), and temperature (T).
[b]Means significant effects on responding variables. Significant effect of factors indicated at the 0.05 level using *.

3.3.3. Thiamin content

Thiamin is an organo-sulfur compound that has pyrimidine and thiazolium heterocycle and methyl bridge (Fitzpatrick & Chapman, 2020). Initial thiamin content was about 67 µg/100g (mean of all 5 varieties) and this is lower than previous studies, which can be due to the protocol used where several steps to deactivate enzymes by heat cause decomposition of the thiamin (Alajaji & El-Adawy, 2006), specifically the attached phosphate groups (Andac-Ozturk et al., 2022).

Thiamin content decreased for Crown, Royal, Orion, Sierra, and Frontier varieties under different storage conditions (Figure 3.5). All variables individually contributed to the decrease in thiamin significantly ($p \leq 0.05$) in Crown and Frontier, whereas the interaction of these variables collectively influenced thiamin levels across all varieties. Significant variation in thiamin concentration occurred due to relative humidity (RH) in the Royal variety, whereas in Orion, both temperature (T) and RH played pivotal roles in determining its concentration. Additionally, in the Sierra variety, thiamin content fluctuated according to the influence of the day. The lowest value for thiamin was for Crown (34.58 µg/100g) under the HTHRH condition after 360 days of storage. The thiamin reduction from the control (0) to 360 days for HTHRH in different varieties was 55%, 38%, 41%, 14%, and 10% for Crown, Royal, Orion, Sierra, and Frontier, respectively. Storage length with lower RH had the highest effect on thiamin content because LTLRH had the lowest values for Sierra (40.7 µg/100g), and Frontier (37.5 µg/100g) compared to harsh storage conditions. In Sierra and Frontier, thiamin content decrease was not affected by temperature and RH significantly, but day had significant effect on thiamin decrease that

might effected thiamin content in LTLRH. Also, LTLRH did not have significant difference compared to HTHRH in Sierra.

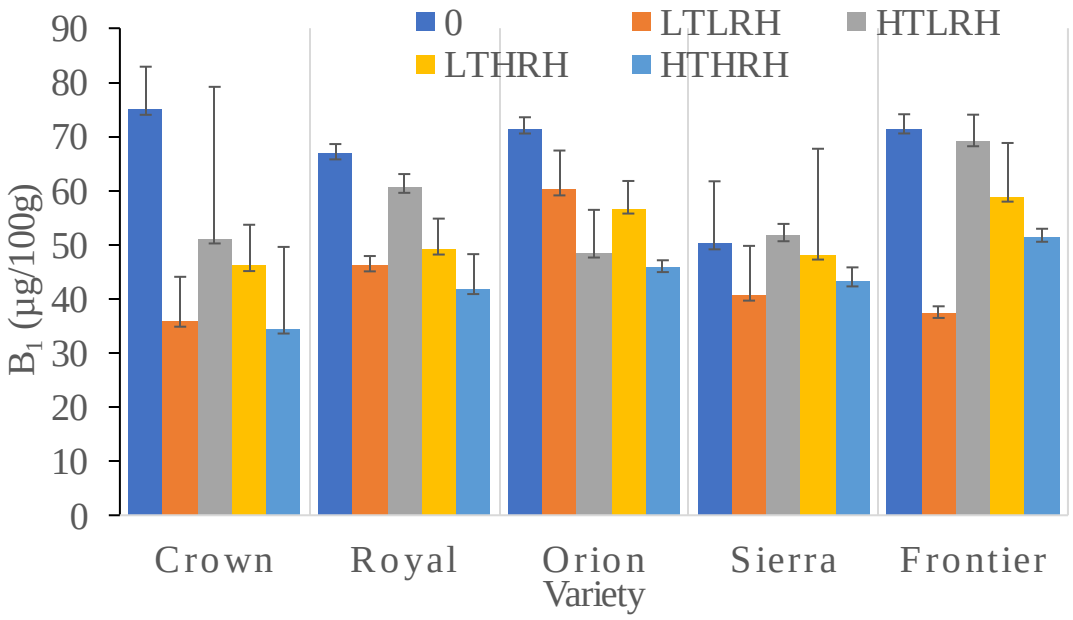

Figure 3.5. Vitamin B_1 (thiamin), changes in different varieties (Crown, Royal, Orion, Sierra, Frontier) in different treatments (0, LTLRH, HTLRH, LTHRH, and HTHRH). Line represents the standard deviation (n =4).

Thiamin is sensitive and reduction can occur during food processing (Kaplan Evlice & Özkaya, 2020), milling (Batifoulier et al., 2006), storage (Godoy et al., 2021), and high temperature and moisture (Ottaway, 2010). The soaking pH had significant effects on thiamin content. The basic and neutral pH led to a decrease in the thiamin content of faba beans and the most stable condition was acidic soaking using citric acid in chickpea and lentil soaking (Prodanov et al., 2004).

3.3.4. Riboflavin content

Sierra, Royal, and Frontier had identical initial riboflavin contents at about 117 µg/100g, whereas Crown had the lowest initial riboflavin content, approximately 93 µg/100g (Figure 3.6). Various factors exerted significant ($p \leq 0.05$) effects on the riboflavin content of different varieties. Specifically, in Crown, riboflavin content declined due to the influence of storage duration and the interaction of various variables. Conversely, in Orion, all variables influenced riboflavin content. The lowest riboflavin content was 22.9 µg/100g in Orion HTHRH treatment, which was significantly ($p \leq 0.05$) lower than the initial content. The riboflavin content decreased over the storage period from day zero to 360 days across different varieties. Specifically, Crown decreased by 19.4%, while Royal exhibited a reduction of 64% (0 compared to HTHRH). Orion had the most significant decline, with riboflavin content decreasing by 79%. Similarly, riboflavin content decreased by 79% in the frontier variety over the same duration (0 compared to HTHRH). Riboflavin content of 0-day in Crown, Royal, and Orion was not significantly ($p > 0.05$) different from HTLRH and LTHRH. Sierra had a different pattern of riboflavin loss. Riboflavin stored at LTHRH (65 ug/100g) was not significantly different than riboflavin of Sierra stored at HTLRH. The riboflavin concentration in Crown at day 0 was not significantly ($p>0.05$) different than the riboflavin concentration of the HTLRH. The riboflavin concentration in the Sierra and frontier zero-day samples was not significantly ($p>0.05$) different than the riboflavin concentration of the LTHRH.

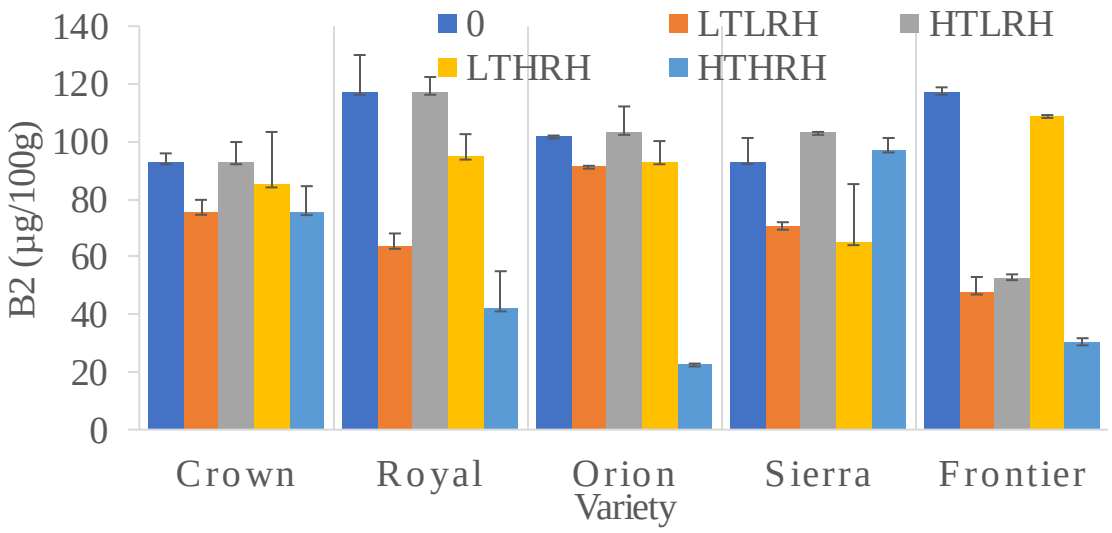

Figure 3.6. Vitamin B_2 (riboflavin), changes in different varieties (Crown, Royal, Orion, Sierra, Frontier) in different treatments (0, LTLRH, HTLRH, LTHRH, and HTHRH). The line represents the standard deviation (n =4).

These findings highlight the variable rates of riboflavin degradation among different varieties during the storage period, underscoring the importance of monitoring and managing storage conditions to maintain nutrient levels. Riboflavin is a yellow-orange organic compound comprising of methylated isoalloxazine core and ribityl side chain which make it an active cofactor. Riboflavin is relatively stable to heat and acidic environments while it is highly sensitive to light and is degraded by reducing agents (Godoy et al., 2021).

The moisture of the environment accelerated the degradation of riboflavin while moisture-free conditions did not affect irradiated riboflavin tablets (Sheraz et al., 2014). Also, thermal degradation of riboflavin increases when temperature and time increase (Astanov et al., 2014). High temperature and RH such as HTHRH treatment could be the reason for the riboflavin degradation in stored samples. Riboflavin is more stable compared to thiamin (Kwok et al., 1998; Sheraz et al., 2014).

3.3.5. Niacin content

Niacin decreased (Figure 3.7) for all varieties from 0 to 360 days of storage under harsh conditions (HTHRH) by 44%, 48%, 8%, 18%, and 25% for Crown, Royal, Orion, Sierra, and Frontier, respectively. The lowest decrease was for the Orion variety, that means harsh conditions (HTHRH= 65% RH, and 40 °C) had the least effect on Orion niacin content. The highest value of niacin was observed in Crown (590.6 µg/100g) at 0-day and the lowest value was detected in Royal (211.7 µg/100g) in HTHRH condition in 360-day sampling. The interaction of the variables affected niacin content significantly ($p \leq 0.05$), excluding Orion. Niacin content decreased dramatically by about 95% in boiled chickpeas (1602 µg/100g to 70 µg/100g) (Alajaji & El-Adawy, 2006). This might be the reason for a lower level of niacin in chickpea samples compared to the literature due to the several steps of boiling and cooling in 5 different steps of enzyme extraction. In boiled or simmered rice, no significant reduction in niacin content was observed. However, degradation of niacin in fortified cookies ranged from 1% to 12%, depending on the temperature and duration of baking (Hrubša et al., 2022). On the other hand, niacin remained stable during the baking of bread (Ottaway, 2010). Niacin contents were not significantly different between Day-0 and LTHRH samples of Royal, Sierra, and Frontier. However, at room temperature and low RH (LTLRH) the niacin concentrations in chickpeas, except Orion, were significantly lower than the niacin from Day-0 samples (Figure 3.7).

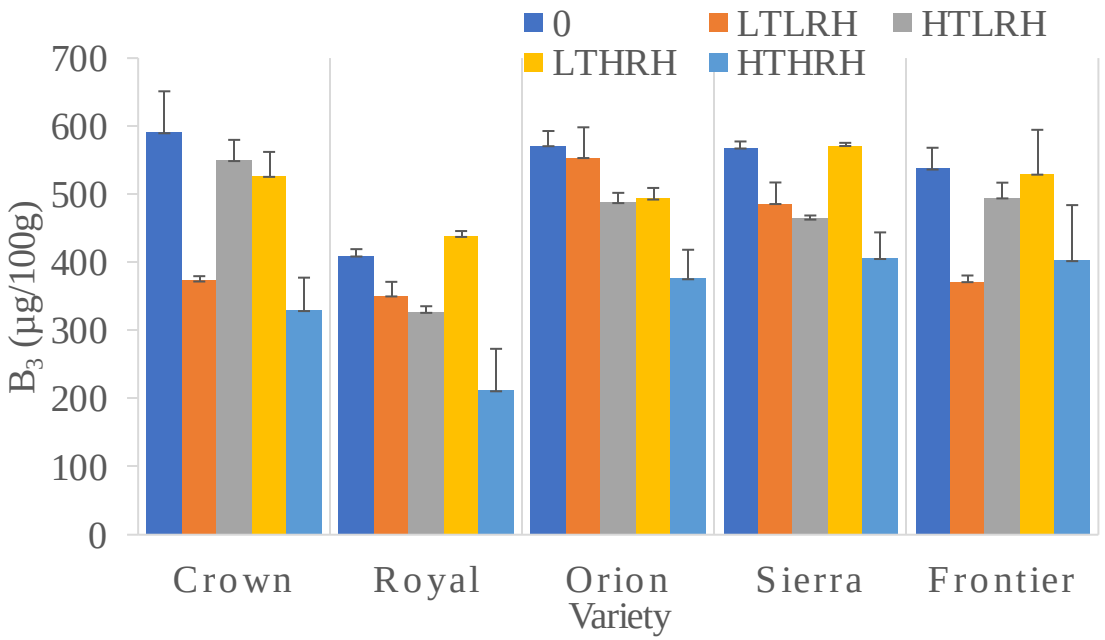

Figure 3.7. Vitamin B_3 (niacin), changes in different varieties (Crown, Royal, Orion, Sierra, Frontier) in different treatments (0, LTLRH, HTLRH, LTHRH, and HTHRH). Line represents standard deviation (n =4).

Niacin is typically resistant to changes in pH, heat, oxygen, and light (Bhalla & Savitri, 2016). However, high temperature and short water-blanch time is better than low temperature and long water-blanch time (Ottaway, 2010). Niacin is stable in the process of heating meat, vegetables, and legumes and leach out to the cooking water causing a reduction of 5-55% of niacin content (Prodanov et al., 2004).

3.3.6. Pyridoxin content

The pyridoxin degrade in highly processed foods by heating and high temperature (Zand et al., 2012). The Pyridoxine content declined over the storage period, and statistical analysis indicated a significant difference ($p \leq 0.05$) in Pyridoxine content between samples collected at 0 days and those collected at 360 days under HTHRH conditions for Crown,

Royal, Orion, and Sierra varieties, with decreases of 5%, 12%, 15%, and 8%, respectively (Figure 3.8). There is no significant decrease ($p > 0.05$) in pyridoxine content between 0 and HTHRH samples of the Orion variety. However, a significant decrease ($p \leq 0.05$) in pyridoxine content was observed between 0 and LTLRH, HTLRH, and LTHRH samples of other varieties.

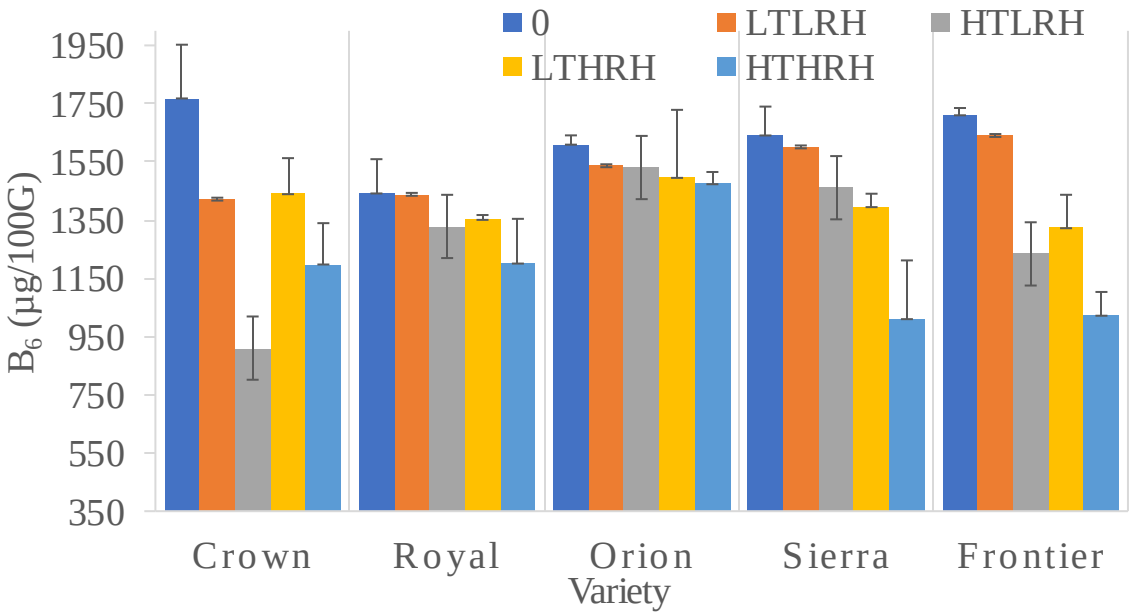

Figure 3.8. Vitamin B_6 (pyridoxin), changes in different varieties (Crown, Royal, Orion, Sierra, Frontier) in different treatments (0, LTLRH, HTLRH, LTHRH, and HTHRH). The line represents the standard deviation (n =4).

In Crown, the LTLRH condition had the lowest values for pyridoxin. This is the same as other vitamins and indicates the effects of the lower RH and storage length on vitamin content. For example, while LTLRH had the lowest value, LTHRH and HTHRH had no significant difference with zero-day. The interaction of the variables had a significant effect ($p \leq 0.05$) on the pyridoxin content of storage samples. The lowest value for B_6 was observed in Crown (910 µg/100g) in HTLRH treatment. Total B_6 in canned red lentils were reported about 350 µg/100g (Andac-Ozturk et al., 2022) and this vitamin is

sensitive to temperature destructing from 20-97% in cooked fish samples (Çatak et al., 2020). Thus, the small decrease in pyridoxin in stored chickpea may be the result of lower storage temperatures compared to boiling temperatures.

3.3.7. Folic acid content

Folate (PteGlu) decreased significantly ($p \leq 0.05$) from 0 to 360 days of storage under HTHRH conditions in all varieties and the most effective factor was the day of storage (Figure 3.9). The folic acid decreased from 0 to 360 days of storage for HTHRH by 58%, 69%, 30%, 39%, and 31% for Crown, Royal, Orion, Sierra, and Frontier, respectively. The highest decreases were detected in Royal. The folic acid content in 29 wild and 4 cultivated lentils revealed that wild lentils had higher folic acid (197-497 µg/100g) concentrations compared to cultivated lentils (174-364 µg/100g) (H. Zhang et al., 2019). Also, the use of tri- or quad-enzyme extraction yielded from 155.8 to 200.9 µg/100g of total folate in soybean (Agyenim-Boateng et al., 2022). Folic acid content for Crown, Royal, Orion, and Frontier at zero day had no significant difference with LTHRH. LTHRH was significantly ($p \leq 0.05$) higher than values for LTLRH in mentioned varieties.

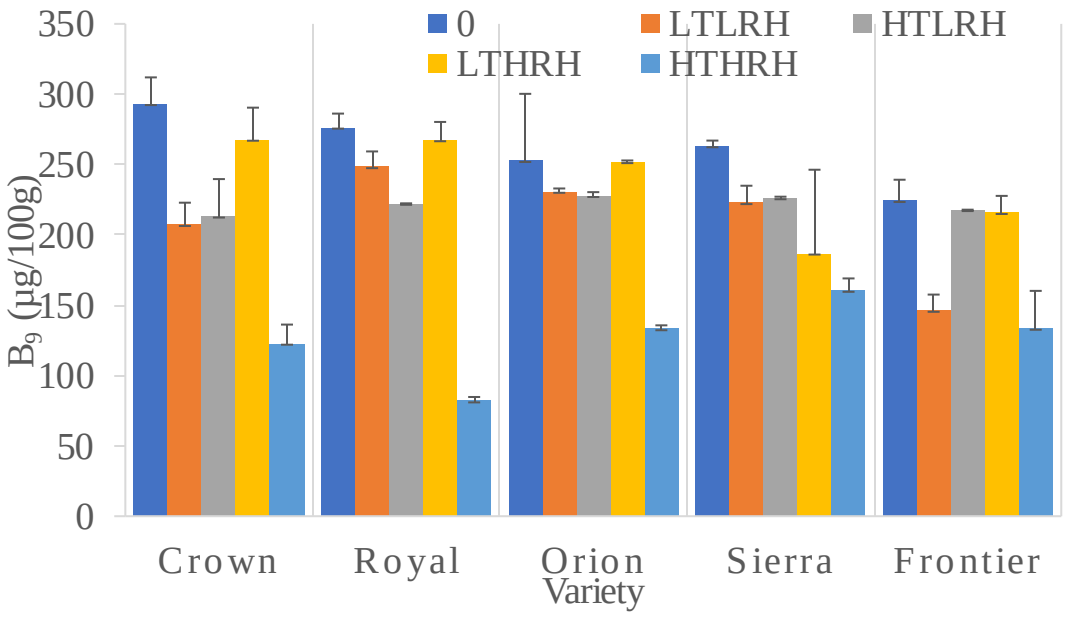

Figure 3.9. Vitamin B_9 (folic acid), changes in different varieties (Crown, Royal, Orion, Sierra, Frontier) in different treatments (0, LTLRH, HTLRH, LTHRH, and HTHRH). The line represents the standard deviation (n = 4).

Humans cannot synthesize folates and uptake of that depends on dietary sources and biofortification of this vitamin helps to increase natural availability (Agyenim-Boateng et al., 2022) although plant folates are present in low concentration and in heterogeneous form (mono and poly-glutamates) (H. Zhang et al., 2018). The highest folate yield in soybeans was observed at pH 5.5 and 150 µL of rat serum in quad-enzyme extraction and that means the stability of folate was high at that pH (Agyenim-Boateng et al., 2022). The incubation with rat plasma hydrolyzes $PteGlu_3$ (Pteroyl-L-triglutamic acid) to PteGlu (Pteroyl-L-monoglutamic acid) completely after 4 h and background noise were decreased by increasing the amount of protease (G. F. Zhang et al., 2005). PteGlu is the most stable vitamer of folates and it was detected even after 100 ℃ heating treatment and it was stable to pH changes (H. Zhang et al., 2018).

3.4. Conclusion

The results support the significant impact of storage conditions on the levels of essential B vitamins such as thiamin, riboflavin, niacin, pyridoxine, and folic acid in stored chickpea. Storage factors such as temperature and RH effect vitamin B levels. Also, storage length plays a vital role in ensuring high quality of chickpea. Harsh storage conditions such as high RH (65%) and high temperature (40 °C) had significant effects on functional properties of chickpea flours while it is indicated that dry environment along with storage time had the highest effects on B vitamin in stored 5 varieties (Crown, Royal, Orion, Sierra, and Frontier) of chickpea for 360 days. Storage length with lower RH had the highest effect on thiamin and riboflavin content of Crown and Sierra. HTHRH also had lowest values for niacin, pyridoxin and folic acid compared to 0-day samples across all varieties. The findings underscore the vulnerability of these vitamins to degradation over time, with different varieties having different rates of decline in B vitamins. Additionally, the importance of monitoring and managing storage conditions was observed to preserve the nutritional integrity of chickpea varieties, particularly considering their growing importance as plant-based protein sources.

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General Conclusions

The investigations into the effects of storage conditions on chickpea composition, functionality, and nutritional integrity underscore the critical role of temperature and relative humidity in storage. The studies revealed significant impacts on moisture content, protein and starch structural features, fat degradation, functional properties, color parameters, and levels of essential B vitamins. Results indicated that major changes in functional and chemical properties started from day 180 of storage under harsh condition. Storage length over 180 days, high temperature (40 °C), high RH (55% and 65%), and combination of these factors are not recommended for chickpea (Crown, Royal, Sierra, Orion, and Frontier) varieties storage condition. For instance, nutritional value had no significant decrease over 360 days in all varieties this means protein and starch content which is important for functional properties. Also, samples after 360 of storage under harsh condition (65% and 40 °C) indicated significant changes. For example, foaming and emulsion capacity decreased for all verities significantly after 180 days and the most effective factor was time of storage while, WHC and OHC had different pattern for Frontier. This indicated that WHC increased after 180 days under harsh condition and then deceased after 360 days. This can be important day to use stored Frontier after 180 days in food applications. Color changes can be good indicator of seed damage under harsh conditions. For example, Royal and Orion samples indicated darker color (brown) after 360 days under harsh condition. Also, despite of having same nutritional factors after 360 days under harsh condition, protein fraction band disappeared and showed low solubility of protein aggregates. Vitamin B decreased after 360 days of storage under various conditions and to maintain high vitamin contents storage length and factors such as

temperature and RH need to be controlled. It was evident that storage management strategies are necessary to mitigate adverse changes and preserve chickpea quality over prolonged periods. The findings emphasized the importance of monitoring and managing storage environments to maintain the nutritional value and functionality of chickpeas, thereby ensuring their continued significance as essential plant-based food sources.

Recommendation and Further research

It is suggested that to observe impact of storage factors, other legumes such as lentil, and pea can be used for further investigations. Storage for more than one-year needs to be investigated for chickpea varieties. Hence, these varieties were all sourced from the northern region of America. It is crucial to investigate additional types and varieties to thoroughly understand the impact of storage factors on the chemical and functional properties. Frontier can be used for protein isolation because it has higher protein content compared to other varieties. Frontier showcased distinct variations in its functional properties. For instance, Frontier stored for 180 days under harsh conditions (65% humidity and 40°C temperature) exhibited attributes suitable for applications requiring high Water Holding Capacity (WHC) and Oil Holding Capacity (OHC). Additionally, Water Absorption Index (WAI) demonstrated a notable increase for all variants after 360 days under harsh conditions, indicating potential suitability for food applications necessitating substantial moisture retention. Another important factor about protein structure, are evaluation of the amino acid profile and effects of the storage factors on essential amino acids and also, digestibility of the proteins can be evaluated. However, it is worth noting that B Vitamin degradation was observed across all treatments following 360 days of storage. Therefore, it is imperative to determine the point at which the effects of storage

factors commence to diminish vitamins in stored chickpeas. Also, different variety investigation is needed, and researchers can identify genetic factors contributing to resilience or vulnerability to storage-induced changes, informing breeding efforts to develop varieties with enhanced storage stability. Furthermore, storage-induced changes on the sensory attributes, cooking properties, and processing characteristics of chickpeas and derived products needs to be observed. This could involve sensory evaluation studies, texture analysis, and evaluation of end-product quality to assess the practical implications of storage conditions on consumer acceptance and product formulation.