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NUTRITIONAL QUALITY AND HEALTH BENEFITS OF CHICKPEA (*CICER ARIETINUM* L.): A FOCUS ON PROTEIN CONTENT

TIPO TESI: sperimentale

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To my family,
my better half,
and to a precious soul
who watches over us.

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ABSTRACT

Chickpea (*Cicer arietinum* L.) is a self-pollinated, annual diploid (2n=2x=16) legume species, with a relatively small genome size (~740Mbp). It is one of the most cultivated legume crops and a potential source of proteins with desirable nutritional and functional properties. Knowledge and consumption of plant proteins should be encouraged for both healthy and sustainability reasons. The main aim of this work was to develop an efficient and rapid protocol to optimize the total protein extraction from chickpea seeds to be used to phenotype, for such trait, large collection of different genotypes.

The first step was to obtain a fine powdered tissue from chickpea seeds; two grinding methods have been compared consisting in the use of mortar and pestle and a TissueLyser, respectively. In both cases low temperature was guaranteed by the use of liquid nitrogen. A trichloroacetic acid (TCA) in acetone extraction method and a phenol extraction method have been, then, compared to optimize protein extractions from chickpea flours. SDS-PAGE analysis has been employed to identify protein subunits based on their molecular weights. Many electrophoretic analyses with gels at different polyacrylamide concentrations and loaded with different protein amounts have been performed to identify the method that gives the best protein resolution. The results showed that the most efficient method in term of time saving and protein extract's quality consists in: i) grinding with mortar and pestle in liquid nitrogen; ii) TCA/Acetone extraction; iii) SDS-PAGE analysis with AnyKd precast gel (Biorad®) by loading samples containing 15µg proteins.

Six chickpea genotypes (four *Kabuli*, two *Desi*) have been analysed with this method to detect if differences in protein content and profiles exist among the diverse chickpea genotypes. The SDS-PAGE analysis showed that the most abundant proteins in chickpea seeds have molecular weights in the ranges 20-25 kDa, 35-40 kDa and 50-70 kDa. They correspond mainly to globulins (11S legumin; 7S vicilin) and albumins, which are the most concentrated proteins in chickpea. Minor protein fractions correspond to glutenin (110 and 55 kDa), lipoxygenase (96 kDa) and prolamin. Protein content seems to be higher in Kabuli types than Desi. In conclusion, the optimized method described in this work can be applied to effectively analyse the protein profile in large collections of chickpea seeds, and for further analyses like Western blot, chromatography and MS spectrometry. Moreover, even if only six genotypes were tested it was possible to highlight some differences in terms of protein content and profiles.

RIASSUNTO

Il cece (*Cicer arietinum* L.) è una specie autogama, annuale e diploide (2n = 2x = 16), caratterizzata da un genoma relativamente piccolo (~740 Mbp). È una delle leguminose più coltivate e rappresenta una potenziale fonte di proteine con importanti proprietà nutrizionali e funzionali. Studi focalizzati sull'analisi delle proteine vegetali e sul loro consumo dovrebbero essere incoraggiati perché migliorano la salute e la sostenibilità ambientale. Il principale obiettivo di questo lavoro è stato lo sviluppo di un protocollo rapido ed efficiente che ottimizzasse l'estrazione delle proteine totali da semi di cece, che possa essere utilizzato per fenotipizzare questo carattere nutrizionale in collezioni ampie di diversi genotipi di cece.

Il primo esperimento è stato il confronto di due diversi metodi di macinazione per ridurre il tessuto in una polvere fine; in particolare la macinazione è stata effettuata con mortaio e pestello e con un TissueLyser, mantenendo in entrambi i casi una temperatura al di sotto dello zero (utilizzo di azoto liquido). Successivamente, il metodo di estrazione delle proteine con acido tricloroacetico (TCA) in acetone è stato confrontato con il metodo di estrazione con fenolo. L'analisi SDS-PAGE è stata impiegata per identificare le proteine basandosi sui rispettivi pesi molecolari. Numerose corse elettroforetiche con gel a diverse concentrazioni di poliacrilammide, caricati con campioni a diverso contenuto proteico, sono state effettuate per selezionare il metodo che offrisse la migliore risoluzione delle proteine. Dai risultati ottenuti è emerso che il metodo più efficiente in termini di risparmio di tempo e qualità dell'estratto prevede: i) macinazione dei semi mediante mortaio e pestello in azoto liquido; ii) metodo di estrazione con TCA in acetone; iii) SDS-PAGE con gel "precasted" ''AnyKd (Biorad®)'' caricato con campioni contenenti 15µg di proteine. Il metodo è stato utilizzato per analizzare sei diversi genotipi di cece (quattro appartenenti alla tipologia Kabuli e due alla Desi) al fine di rilevare eventuali differenze a livello di contenuto e profilo proteico. Dalla caratterizzazione in SDS-PAGE è emerso che le proteine più abbondanti nei semi di cece sono comprese tra 20-25 kDa, 35-40 kDa e 50-70 kDa. Queste frazioni corrispondono principalmente a globuline (leguminosa 11S; vicilina 7S) e albumine, che sono le proteine più concentrate nel cece. Le frazioni minori corrispondono a glutenina (110 e 55 kDa), lipossigenasi (96 kDa) e prolamina. Il contenuto proteico sembra essere più alto nei tipi Kabuli rispetto ai Desi. Il metodo sviluppato in questo lavoro può essere utilizzato per analizzare efficacemente il profilo proteico di semi di ampie collezioni di genotipi diversi di cece e per la caratterizzazione mediante ulteriori analisi, quali Western blot, cromatografia e spettrometria MS. Infine, anche se soltanto sei genotipi diversi sono stati utilizzati è stato possibile evidenziare alcune differenze per quanto riguarda il contenuto e il profilo proteico.

CHAPTER I: INTRODUCTION

1.1. LEGUMES

Legumes are plants belonging to the Fabaceae family (or Leguminosae), a large and economically important family of flowering plants, order Fabales. The family is widely distributed and is the third-largest land plant family for number of species, behind only the Orchidaeaceae and Asteraceae, with ~770 genera and ~19,500 species (Lewis *et al*. 2005, 2013; LPWG, 2013; Byng *et al*. 2016)

Several Leguminosae species were domesticated by humans **(Table 1)**, such as soybean, beans, faba bean, pea, chickpea, lentil, peanut, lupine, pigeon pea, mung bean, peanut, cowpea or alfa-alfa, for both food and feed. Almost all the domesticated species belong to the Papilionoideae.

Today, India is the most important producer of legumes in the world, with 25.5 million of tonnes produced in 2018, followed by Myanmar (6.1 mln of tonnes), USA (3.4 mln of tonnes) and Brazil (2.9 mln of tonnes), with a total world production of 92.2 million of tonnes (FAOSTAT, 2018). The most cultivated legume is soybean, with 348 mln of tonnes (FAOSTAT, 2018) followed by beans, peas and chickpea. The most soybean producing countries are Brazil, USA and Argentina. Other legumes grown to a lesser extent are lentils, broad bean and lupines. Bean is mainly produced from Brazil, India and China. About 25 million hectares are used for its production worldwide, with a production of 30 mln of tonnes. Growing peas is distributed homogeneously worldwide, especially in USA and European Union (212 mln of tonnes). Chickpeas are produced principally in India and Pakistan, with a total world production of 131 mln of tonnes (FAOSTAT, 2018).

Scientific name	Common name
Arachis hypogaea L.	Peanut
Cajanus cajan (L.) Millsp.	Pigeon pea
Cicer arietinum L.	Chickpea
Glycine max (L.) Merr.	Soybean
Lens culinaris Medic.	Lentil
Phaseolus lunatus L.	Lima bean
Phaseolus vulgaris L.	Common bean
Pisum sativum L.	Pea
Vicia faba L.	Faba bean
Vigna angularis (Willd.) Ohwi & Ohashi	Adzuki bean
Vigna radiate (L.) Wilczek	Mung bean
Vigna unguiculata (L.) Walp.	Cowpea

Table 1: Here is a list of twelve primary grain legumes destined to human consumption *(*Hymowitz *et al.* 1990)

Crop legumes can be used as forage, grain, bloom, pharmaceutical/industrial, fallow/green manure, and timber species. Most legume crops fill two or more roles simultaneously, depending upon their degree of maturity when harvested. Grain legumes are cultivated for their seeds, which are used for human and animal consumption or to produce oils for industrial uses.

Legumes are consumed all over the world, especially south Asia and Sub-Saharan Africa, where they provide dietary proteins and are therefore vitally important to the population of less developed countries. (Navarro *et al*. 2014). Recently, there is an ever-increasing demand of plant proteins for food by consumers also in developed countries. Moreover, as a result of the increasing applications of plant proteins in food and non-food markets, the production of plant protein isolates represents a growing industry (Yu-Wei *et al*. 2006). Dried legumes are rich in proteins, contain little fat and are excellent sources of dietary fibre and micronutrients, such as iron, zinc, potassium and folate, however, legumes contain some antinutritional factors

that interfere with absorption of some nutritional compounds (Khattaba *et al*. 2009; Sparvoli *et al*. 2015). Examples are phytic acid, tannins, trypsin inhibitors, and oligosaccharides. Treatments like water soaking, boiling, roasting, microwave cooking, autoclaving, fermentation and micronization could reduce drastically antinutritional compounds (Khattaba *et al*. 2009)**.** Legumes are recommended by the World Health Organization for the management of chronic non-communicable diseases such as diabetes and heart disease (Hosseinpour-Niazi *et al.* 2015). The low-fat content and interaction of their sterols have proven effective in maintaining low LDL cholesterol levels and reducing blood pressure (Hosseinpour-Niazi*et al.* 2015). Since legumes contain nutrients similar in quantity and type to those of protein foods and vegetables, they can be considered as a vegetable or as a protein food in order to satisfy the reference assumptions (Dietary Guidelines for Americans, 2015). The consumption of vegetable proteins (mainly represented by dried legumes) should be favoured over the consumption of proteins of animal origin for sustainability reasons. The global livestock sector accounts for 14.5% of anthropogenic greenhouse gas emissions, playing an important role in climate change (Folloni*et al.* 2017). About 6.5 billion people currently live on our planet and, by 2050, this number is expected to increase by almost 50% to over 9 billion. The strong increase in food demand will require a greater consumption of alternatives to animal foods, like ones from plants or insect origin. According to this, legumes could be an important alternative.

1.2. CHICKPEA (*Cicer arietinum* L.)

Chickpea is an annual legume belonging to the order of Fabales, subfamily Fabaceae, tribe Ciceraceae, genus *Cicer*. It is the unique cultivated species of genus *Cicer*. It is a diploid (2n=2x=16; ~740Mbp), self-pollinated species that humans have adapted to various seasonalities around the world, where it is sown following seasonal rains and matures on residual soil moisture. There is now increasing interest in the use of chickpea and other legumes in human consumption; one of the main reasons is the fact that they are important sources of proteins. In this regard, chickpea is characterized by high protein content with high digestibility (Diwakar *et al.* 1995).

1.2.1. GENERAL BOTANY

Chickpea is an herbaceous annual plant which branches from the base. It is almost a small bush with diffused, spreading branches. The plant is mostly covered with glandular or no glandular hairs, but some genotypes do not possess hair (Diwakar *et al*. 1995)

Figure 1*:* Chickpea seeds with different colours

Based on seed size and colour, cultivated chickpeas are of two types **(Figure 1**): i) *Kabuli*, seeds are large (100-seed mass >25g), round or ram head, and cream-colored. The plant is medium to tall, with large leaflets and white flowers, and contains no anthocyanin; ii) *Desi*, seeds are small and angular in shape, colour from cream, black, brown, yellow to green. The plants are short with small leaflets and purplish flowers and contain anthocyanin. Chickpea seeds have a seed coat, two cotyledons, and an embryo (Diwakar *et al*. 1995)

The seed coat consists of two layers, the outer testa and the inner tegmen, and a hilum. The hilum is the point of attachment of the seed to the pod. Chickpea seeds germinate at an optimum temperature (28-33°C) and moisture level in about 5-6 days. Germination begins with absorption of moisture and swelling of the seed (Diwakar *et al*. 1995)

Chickpea plants have a strong taproot system with 3 or 4 rows of lateral roots, stem is erect, branched, viscous, hairy, terete, herbaceous, green, and solid. There are primary, secondary and tertiary branches. Leaves are petiolate, compound, and unimparipinnate. The solitary flowers are in an axillary raceme. Sometimes there are 2 or 3 flowers on the same node. Such flowers possess both a peduncle and a pedicel. Chickpea flowers are complete and bisexual and have papilionaceous corolla. They are white, pink, purple or blue in colour (**Figure 2**). (Diwakar *et al*. 1995).

Pollination takes place before the opening of the bud which is cleistogamous. Thus, self-pollination is the rule. Natural cross-pollination has been reported, but most reports indicate 100% self- pollination (Singh *et al.* 1997).

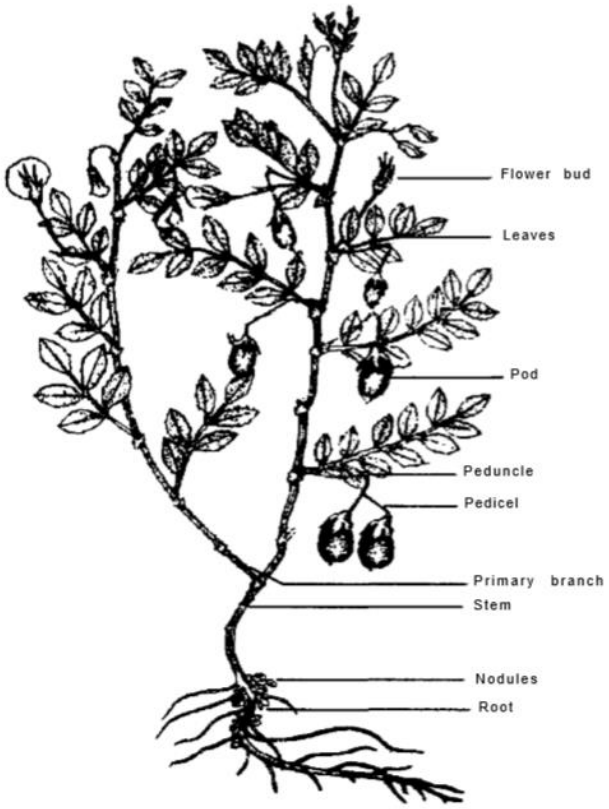

Figure 2: A representation of Chickpea plant.

1.2.2. AGRICULTURAL PRACTICES

Being a legume, chickpea can fix atmospheric N_2, converting it in ammonia (NH_3), which is useful for living organisms on the field. This is possible due to the symbiosis established with bacteria of the genus *Rhizobium*, that settle in the host's roots, inducing the formation of typical root nodules, subtract various organic compounds and mineral salts from the host itself, yielding in exchange nitrogen compounds. Chickpea requires optimum amounts of phosphorus, potassium, sulphur, and other nutrients. The response to nutrient application in chickpea depends on the nutrient status of the soil, agroclimatic conditions, and the genotype. Both organic and inorganic sources of nutrients and *Rhizobium* inoculation have been found to be useful for chickpea growth and yield (Diwakar *et al.* 1995)

There are wide variations in the agroclimatic conditions under which chickpea is grown around the world. These environments differ in photoperiod, temperature, and precipitation. Due to

the variation in longitude, the time of sowing also varies from one region to another. Smithson *et al*. (1985) classified chickpea-growing areas into four major geographical regions:

a) Indian subcontinent b) West Asia, North Africa, and Southern Europe c) Ethiopia and East Africa d) The Americas and Australia.

Chickpea is grown on different types of soils ranging from sandy (Pakistan) to sandy loams (northern India) to deep black cotton soils (central India, West Asia, and the Ethiopian highlands). It is also cultivated on calciferous soils with a subsoil layer of CaCO3 in West Asia. The best soils for chickpea growth are deep loams or silty clay loams devoid of soluble salts (Chandra *et al.* 1970). Such soils retain up to 200 mm moisture in the soil profile up to a depth of 1m (Saxena *et al.*1979). The maximum nutrient availability from the soil is at a pH range of 5.7 to 7.2. Chickpea requires good soil aeration. Chickpea is highly sensitive to salinity in the soil, which has an adverse effect on dry-matter production and uptake of phosphorus, zinc, and iron (Diwakar *et al.* 1995).

Chickpea cropping systems and production practices varies from region to region and within the same region. Often, chickpea is grown in rotation within other crops, due to its ability to fix atmospheric N_2 on the field. Crop rotation with chickpea includes those depleting plants (e.g. maize, sugarcane, wheat etc.) that reduce the soils from certain nutrients and selects for a highly competitive pest and weed community. In the alluvial soils of the Indo-Gangetic plain, chickpea follows rainy-season crops in double-cropping systems. In sandy soils in northern India and in Pakistan, it is grown either after pearl millet or after fallow when monsoon rains are insufficient. In central and peninsular India, chickpea is also grown in rotation with sugarcane and maize. In west Asia, Northern Africa and Southern Europe, in areas where the rainfall is more than 400 mm, chickpea is grown with cereals such as wheat and barley and with melons, sesame, and forage crops. In the drier regions, a chickpea-fallow-winter cereal crop rotation is followed. Ethiopia and the East African Highlands are at elevations ranging from 1400 m to 2300 m. Mostly Desi chickpea is cultivated here in rotation with barley and wheat on conserved soil moisture at the end of the rainy season. In central America and Australia, chickpea is grown in rotation with maize, sesame, wheat, or one or more years of pasture.

The most serious disease affecting chickpea is rabies or anthracnose (*Ascochyta rabiei)*, which can result in the destruction of cultivation. Against this disease the major strategy is the development of resistant varieties; some results are obtained with direct control based on seed tanning and spraying at the beginning of the pod formation. Other fungi that can cause damage

are chickpea rust (*Uromyces cicer-arietini*), wilting, caused by *Rhizoctonia spp*., *Fusarium* spp. *Verticillum* spp.

The most serious attacks of insects are brought by the *Heliotis armigera* on the pods, larvae of *Liriomyza cicerina minatrice* of the leaves, and *Callosobruchus chinensis* which attacks the seeds in the warehouse.

1.2.3. HISTORY AND ECONOMY

Chickpea was one of the first domesticated crops together with other species like wheat, barley, lentil, flax or vetch in a small area of the southwest of Turkey. The crop evolved from a unique ancestor, *Cicer reticulatum*. Diversity is exceedingly low in the modern gene pool, owing to successive human-induced bottlenecks. Archaeological evidence indicates that during the Bronze Age the crop was well established in the Near East, spreading subsequently to Bulgaria, Greece and eventually southern Europe. Chickpea was among the first agricultural crops introduced to Europe via the Black Sea. Early in its cultivation history, chickpea was introduced to India and subsequently over the Mediterranean basin to the east of Africa, though the Nile River. Chickpea crossed the Atlantic Ocean with early colonial activity (Redden *et al.* 2006). Changes with domestication initially included loss of dormancy, reduced pod dehiscence, larger seed size, larger plant size and variants with more erect habit and reduced anthocyanin pigmentation. The key to chickpea domestication was the change from a winter habit with an autumn sowing to a spring habit, which avoided or reduced the threat of lethal infestation of endemic ascochyta pathogen complex (Redden *et al*, 2006).

Chickpea is one of the most important leguminous crops grown for human consumption. In 2018, world production of chickpeas increased by 16% over 2017 levels, rising to a total of 17.2 million tonnes. India alone accounted for the 66% of the entire world production, while Australia, United States, and Turkey were secondary producers (FAOSTAT, 2018). India, Bangladesh, and Pakistan were the top importers of chickpeas in 2016, whereas Australia, Russia, and Canada were the top exporters of chickpeas that same year (FAOSTAT, 2018).

1.2.4. BIOCHEMICAL AND PHYSICAL CHARACTERISTICS

The nutritional quality of chickpea seeds can vary depending on the environment, climate, soil nutrition, soil biology, agronomic practices, stress factors (biotic and abiotic) and industrial processing including the influence of cooking methods on the nutritional quality and development of attractive, convenient ready-to-eat food formulations. The cotyledon and embryo make up most of the nutritionally beneficial parts of the seeds while the seed coat contains many of the antinutritional factors (Yadav *et al.* 2007; Summo *et al.* 2019). According

to Hulse *et al.* (1989), chickpea seeds typically contain 29% protein, 59% carbohydrate, 3% fibre, 5% oil and 4% ash. Wang *et al.* (2004) compared the chemical composition of iranian kabuli- and desi-type chickpeas grown in East Azerbajan and Iran **(Table 2)**. Dry and organic matter, ether extract, total phenolic compound and starch showed negligible differences, while crude protein, total tannin, non-fibrous carbohydrate and soluble sugar showed minimal differences. Crude fibre and neutral detergent fibre are very different and higher in desi type.

Constituents	***Kabuli***	***Desi***	**Difference**
Dry matter	92.08 %DM basis	91.17	NS
Organic matter	97.84	97.15	NS
Crude protein	24.63	22.76	*
Crude fibre	6.49	9.94	**
Neutral detergent fibre	16.70	20.47	**
Ether extract	7.38	7.11	NS
Total tannin	0.09	0.125	*
Total phenolic compound	0.270	0.265	NS
Non-fibrous carbohydrate	49.13	46.81	*
Starch	39.12	38.48	NS
Soluble sugars	8.43	7.53	*

NS: Non Significant; *: $p < 0.05$; **: $p < 0.01$

Table 2: Chemical composition of kabuli and desi types of chickpea 3on dry matter basis (%)

1.2.4.1 Energy value, carbohydrates and lipids in chickpea

Energy values for chickpea have been reported at 334-437 Kcal/100g for Desi type and 357-446 Kcal/100 for Kabuli type. The Kabuli type generally have slightly higher energy values than Desi type grown under identical conditions due to a higher seed coat component of Kabuli cultivars, which are richer in carbohydrates and fat content (Khan *et al*, 1995).

The total carbohydrate content in chickpea is higher than other legumes. Chickpea contains monosaccharides (ribose, glucose, galactose and fructose), disaccharides (sucrose and maltose) and oligosaccharides (stachyose, ciceritol, raffinose and verbascose). The amount of these fractions varies, though not significantly, between the Desi and Kabuli genotypes (Jukanti, Gaur, Gowda, & Chibbar, 2012). Sanchez-Mata *et al.* (1998) reported the following monosaccharides concentrations for chickpea: galactose 0.05g/100g, ribose 0.11g/100g, fructose 0.25g/100g, and glucose, 0.7g/100g. Maltose (0.6%) and sucrose (1–2%) have been reported to be the most abundant free disaccharides in chickpea. α-Galactoses are about 62% of total sugar (mono-, di- and oligosaccharides) content in chickpea. The two important groups

of α-galactoses present in chickpea are: raffinose family of oligosaccharides, including raffinose (trisaccharide), stachyose (tetrasaccharide) and verbascose (pentasaccharide), and galactosyl cyclitols, including ciceritol. Ciceritol and stachyose constitute 36–43% and 25%, respectively, of total sugars (mono-, di- and oligosaccharides) in chickpea seeds (Sanchez-Mata *et al*. 1998).

Starch is the major storage carbon reserve in pulse seeds (Chibbar *et al*. 2010). The content of starch varies from 41 to 50% of the total carbohydrates, with the Kabuli type having more soluble sugars (sucrose, glucose and fructose) compared with the Desi type. The total starch content of chickpea seeds has been reported to be about 525g/kg of dry matter, with about 35% of which being resistant starch (resistant to digestive enzymes and digestion, thus, not absorbed in the small intestine of healthy individuals) and the remaining 65% was available (enzymatically digested in the small intestine) starch (Singh *et al.* 1980). This finding was consistent with those observed in other studies (Jood *et al.* 1998; Chung *et al.* 2008; Aguilera *et al.* 2009). According to Aguilera *et al.* (2009) changes in starch, functional, and microstructural characteristics occurred in chickpea under soaking, cooking, and industrial dehydration processing. Available starch in raw legumes represented 57-64%, and resistant starch (RS) is a significant component. As a result of cooking, available starch contents of soaked chickpea significantly increased (21 and 12%, respectively) and RS decreased (65 and 49%, respectively) compared to raw flours. A similar trend was observed by dehydration, (73% of RS decrease).

Dietary fibre is the indigestible part of plant food in the human small intestine. It is composed of poly/oligosaccharides, lignin and other plant-based substances. They can be classified into soluble and insoluble fibres. Aguilera *et al*. (2009) reported a total dietary fibres of Spanish chickpea cultivars based on enzymatic digestion of non-dietary fibers, equal to 18–22g/100g of raw chickpea seed. Soluble and insoluble dietary fibres are about 4–8 and 10–18g/100g of raw chickpea seed, respectively. (Aguilera *et al.* 2009). Dalgetty & Baik (2003) reported a fibre content of chickpea hulls on a dry weight basis lower (75%) compared with lentils (87%) and peas (89%). The lower dietary fibres in chickpea hulls can be attributed to the difficulty in separating the hull from the cotyledon during milling. The Desi type have a higher total dietary fibres and insoluble dietary fibres compared with the Kabuli type, this could be due to thicker hulls and seed coat in the Desi type (11.5% of total seed weight) compared with the Kabuli type (only 4.3–4.4% of total seed weight) (Rincon *et al.* 1998).

The total fat content in raw chickpea seeds varies from 2.70 to 6.48% (El-Adawy *et al.* 2006). Desi-type chickpea varieties show lower values (2.95g/100g) for crude fat content (Shad, Pervez, & Zafar, 2009). The fat content in chickpea (6.04g/100g) is higher than that in other legumes such as lentils (1.06g/100g), red kidney bean (1.06g/100g), mung bean (1.15g/100g) and pigeon pea (1.64g/100g), and also in cereals such as wheat (1.70g/100g) and rice (about 0.6g/100g) (USDA; 2010) Chickpea is composed of about 66% of poly-unsaturated fatty acids (PUFA), about 19% of mono-unsaturated fatty acids (MUFA) and about 15% of short-chain fatty acids (SFA). On average, oleic acid (OA) was higher and linoleic acid (LA) was lower in the Kabuli type compared to the Desi type (Singh *et al.* 1985). Chickpea is a relatively good source of nutritionally important PUFA, LA (51.2%) and monounsaturated OA (32.6%). Chickpea has higher amounts of LA and OA compared with other edible pulses such as lentils (44.4% LA; 20.9% OA), peas (45.6% LA; 23.2% OA) and beans (46.7% LA; 28.1% OA) (Daun *et al.* 2004). LA is the dominant fatty acid in chickpea followed by OA and palmitic acid. Chickpea cannot be considered as an oilseed crop since its oil content is relatively low (3.8–10%) (Singh *et al.* 1985) in comparison with other important oilseed pulses such as soybean or groundnut. However, chickpea oil has medicinal and nutritionally important tocopherols, sterols and tocotrienols (Zia-Ul-Haq *et al.* 2007). Sitosterol (72.52–76.10%) is the dominant sterol in chickpea oil followed by campesterol. However, the a-tocopherol content in chickpea is relatively higher (8.2mg/100g) than other pulses such as lentils (4.9mg/100g), green pea (1.3mg/100g), red kidney bean (2.1mg/100g) and mung bean (5.1mg/100g) (USDA, 2010).

1.2.4.2 Proteins in chickpea

Protein content

Most legumes have high nitrogen contents, due to their ability to fix atmospheric nitrogen through a symbiotic association with soil microbes. The protein concentration of chickpea seed ranges from 16.7% to 30.6% for Desi and 12.6% to 29.0% for Kabuli type, respectively, and is commonly 2-3 times higher than cereal grains. (Shyam, Yadav *et al.* 2007).

The major proteins found in most legumes are globulins (legumin, vicilin) and albumins (including enzymatic proteins, protease inhibitors, amylase inhibitors and lectins). Minor proteins include prolamins and glutelin usually associated with cereal grain protein. Globulins and albumins are salt and water soluble, respectively, whereas prolamins are alcohol soluble and glutelin are acid and base soluble. (Patto *et al.* 2014). According to Singh *et al.* (1982), globulin comprise the major seed storage protein (56.0%), followed by glutelin (18.1 %),

albumin (12.0%), and prolamin (2.8%). Globulins represent about 70% of legume seed proteins and are composed of two major groups, characterized by their sedimentation coefficients, the 11S (320-400 kDa) or legumin, and the 7S (145-190 kDa) or vicilin (Casey et aI., 1993). The albumin fraction is more heterogeneous and is more abundant in sulphur amino acids comparing to the globulin fraction, thus the albumins in legume proteins may naturally complement the amino acid pattern of globulin. In general, most albumin proteins have some physiological functions, such as enzymatic activity of lipoxygenases, glycosidases, or proteases involved in the degradation of storage proteins. Other albumins, such as protease inhibitors or lectins, are implicated in defensive mechanisms. The name "Prolamin" was originally based on the observation that these proteins are generally rich in proline and amide nitrogen derived from glutamine. Prolamins are generally defined as soluble in alcohol/water mixtures, but some prolamin occur as alcohol-insoluble polymers.

Amino acidic profile

The amino acid profile in chickpea is well balanced, apart from the limited sulphur amino acids (methionine and cysteine, which are essential amino acids that human requires from diet due to its inability to synthetize by itself) and the high content in lysine. Hence, chickpea is an ideal companion to cereals, which are known to be higher in sulphur amino acids but limited in lysine (Shyam, Yadav *et al*. 2007). Commonly consumed food pulses such as chickpea, field pea, green pea, lentils and common beans have about 1.10g/16g N of methionine and cystine. No significant differences in the amino acid profiles of Kabuli- and Desi-type chickpeas were reported (Daun *et al*. 2004).

Amino acid	MPI[d]	IPI[d]	FAO/WHO/UNU Rererence protein		
			Infant	Preschool child	Adult
Essential					
Lysine	6.1	6.8	6.6	5.8	1.6
Threonine	3.4	3.1	4.3	3.4	0.9
Valine	4.3	3.9	5.5	3.5	1.3
Methionine	1.5	1.9	4.2[b]	2.5[b]	1.7[b]
Cysteine	2.0	2.2			
Isoleucine	4.3	4.0	4.6	2.8	1.3
Leucine	8.0	8.0	9.3	6.6	1.9
Phenylalanine	3.7	4.7	7.2[c]	6.3[c]	1.9[c]
Tyrosine	2.8	2.6			
Histidine	2.1	2.4	2.6	1.9	1.6
Subtotal	38.2	39.6			
Non-essential					
Arginine	11.1	9.3			
Aspartic acid	11.7	11.5			
Serine	5.5	5.6			
Glutamic acid	18.3	17.4			
Proline	1.9	4.1			
Glycine	3.0	3.7			
Alanine	5.0	3.9			
subtotal	56.5	55.5			

[a]Mean of two determinations. Values changed from 2 to 9.6% in relation to mean; average variation 4.1%. Tryptophan not determined.
[b]Requirements for methionine ± cysteine.
[c]Requirements for phenylalanine ± tyrosine.
[d]MPI = Micelle protein isolate; IPI = Isoelectric protein isolate.

Table 3: Amino acid composition of chickpea protein isolates (g/100g protein). Source: Pareds-Lòpez *et al.* (1991)

A comparison of amino acid profile of two chickpea protein isolates (obtained by micellization vs IE precipitation) from defatted chickpea flours *cv. Athenas* (Sevilla, Spain) is showed in **Table 3**. Both samples have high amounts of arginine aspartic and glutamic acids; the total amount of these three amino acids ranged from 38.2 to 41.3 g/100 g protein. In relation to the FAO/WHO/UNU (1985) reference pattern for infants, for Micelle-protein isolate (MPI) all amino acids were in lower amounts than requirements and for Isoelectric-protein isolates (IPI) only three amino acids (lysine and phenylalanine + tyrosine) met the required levels. The data also indicated that chickpea proteins contained adequate amounts of most essential amino acids for preschool children and all essential amino acids for adults **(Table 2)**. There are slight variations in the amino acid patterns of the two isolates. Source: Paredes-Lòpez *et al*. (1991).

Functional properties

Chickpea has been considered as a source of relatively high-quality protein with desirable functional properties (Hulse, 1989). Chickpea proteins have noticeable solubility, water

absorption, fat absorption, emulsion capacity and foam stability properties (Paredes-Lopez *et al.* 1991; R Sanchez-Vioque *et al*. 1999).

The *in vitro* protein digestibility of raw chickpea seeds varies from 34 to 76% (Khalil, Zeb,, & Mahood, 2007). Chitra *et al*. (1995) found higher *in vitro* protein digestibility values for chickpea genotypes (65.3–79.4%) compared with those for pigeon pea (*Cajanus cajan*; 60.4–74.4%), mung bean (*Vigna radiata*; 67.2–72.2%), blackgram (*Vigna mungo*; 55.7–63.3%) and soybean (*Glycine max*; 62.7–71.6%). The digestibility of protein from the Kabuli type is higher than that from the Desi type (Singh et al. 1982; Paredes-López et al. 1991; Miao et al. 2009).

The main physicochemical properties of chickpea proteins were investigated by Sanchez-Vioque *et al.* (1999) and showed in **Table 4**. Two protein isolates extracted by alkaline extraction (Isolate A without sodium-sulphite and Isolate B with sodium-sulphite) from chickpeas cv. *Athenas* (Sevilla, Spain) were analysed and compared with chickpea flour (CF). Marked differences in protein solubility, water and fat absorption and emulsion capacity were observed between Isolate-A and B. Isolate-A showed a higher water and fat absorption than Isolate-B whereas this latter was more soluble and had a better emulsion capacity.

	CF	IA	IB
Solubility[a]	31.8 ± 1.1	26.6 ± 0.9	46.3 ± 3.2
Water absoportion[b]	178.8 ± 2.4	343.7 ± 30.1	199.5 ± 4.9
Fat absorbtion[c]	135.8 ± 6.1	409.4 ± 24.9	125.7 ± 11.2
Emulsion capacity[d]	94.7 ± 0.7	48.1 ± 5.7	76.9 ± 2.2

[a] Percentage of soluble nitrogen in 0.1 M NaCl solution at pH 7.
[b] Grams in water absorbed per 100g sample.
[c] Grams of fat absorbed per 100g sample.
[d] Percentage of fat emulsified (% weight).

Table 4: Functional properties of chickpea protein isolates analysed at pH 7. Chickpea seeds cv. Athenas are colletected from Koipesol Semillas, S.A. (Sevilla, Spain) Source: Paredes-Lòpez *et al.* (1991).

Protein solubility is one of the most important functional properties of commercial food protein preparations. Solubility can affect the thermodynamic reactions of the equilibrium between protein-protein and protein-solvent interactions under a given set of environmental conditions, and is related to the net free energy change arising from interactions of hydrophobic and hydrophilic residues of the protein with the surrounding aqueous solvent; it can be affected by pH, ionic strength, ion types, temperature, solvent polarity, and processing conditions (Damodaran *et al*. 1996).

Water absorption capacity of isolates may be affected by conformation and environmental factors; conformational changes in the protein molecules may expose previously enclosed amino acid side chains, thereby making them available to interact with water. Oil absorption of food products is an important functional property because it improves mouthfeel and flavour retention. Proteins constitute an important group of emulsifiers (e.g. soy lecithin) because they cause reduction of interfacial tension, form rigid interfacial films and possess charged groups (Paredes-Lòpez *et al.* 1991).

1.2.4.3 Micronutrients

Vitamins

Chickpea contains several water-soluble vitamins such as B vitamins and vitamin C, as well as several lipid-soluble vitamins such as vitamin A (found as provitamin A carotenoids), vitamin E (tocopherols and tocotrienols) and vitamin K. (Jukanti *et al.* 2012). Chickpea is a relatively inexpensive and good source of folic acid and tocopherols. It is a relatively good source of folic acid coupled with more modest amounts of water-soluble vitamins such as riboflavin (B2), pantothenic acid (B5) and pyridoxine (B6), and these levels are liked or higher than those observed in other pulses. However, niacin concentration in chickpea is lower than that in pigeon pea and lentils. (Jukanti *et al.* 2012). In **Table 5**, chickpea vitamin content detected by different studies is compared. Chavan *et al.* (1986) and Ciftci *et al.* (2010) studied vitamin composition of Indian chickpea cultivars. Wang & Daun (2004) compared vitamin composition of Desi and Kabuli iranian cultivars. USDA (2010) defined chickpea viramin content of Kabuli type.

Vitamins	Chavan et al.*	Wang & Daun**		Ciftci et al.***	USDA
		K	D		K
Retinol (A)	-	ND	ND	-	ND
Vitamin C	2.15 – 6.00	1.34	1.65	-	4.0
Vitamin (D_2 + D_3)	-	ND	ND	115.4	ND
Thiamin (B_1)	0.028 – 0.40	0.4	0.29	-	0.477
Riboflavin (B_2)	0.15 – 0.30	0.26	0.21	-	0.212
Niacin (B_3)	1.6 – 2.90	1.22	1.72	-	1.541
Pantothenic acid (B_5)	-	1.02	1.09	-	1.588
Pyridoxine (B_6)	0.55	0.38	0.30	-	ND
Cyanocobalamin (B_{12})	-	ND	ND	-	0.535
Biotin	-	ND	ND	-	-
γ-Tocopherol	-	10.68	9.33	6.9	-
α-Tocopherol (vit. E)	-	2.24	1.91	22.0	0.820
Choline, total (μg/100g)	-	-	-	-	95.20
Folic acid	150.0	299.21	206.48	-	557.00
Vitamin A, retinol activity equivalent (RAE)	-	-	-	-	3.00
β-carotene	-	-	-	46.3	40.00
Vitamin K (phylloquinone)	120.0	-	-	23.2	9.00

K, Kabuli; D, Desi; USDA, United States Department of Agriculture; ND, measured but not detected.
*Expressed as mg/100g
** The type of chickpea is not specified
***Expressed as μg/100g.

Table 5: Vitamins composition of chickpea seeds obtained from different studies mentioned in Jukanti *et al.* (2012).

Minerals

Raw chickpea seeds on an average provide about 5.0mg/100g of Fe, 4.1mg/100g of Zn, 138mg/100g of Mg and 160mg/100g of Ca; about 100g of chickpea seeds can meet the daily dietary requirements of Fe (1.05mg/d in males and 1.46mg/d in females) and Zn (4.2mg/d in males and 3.0mg/d in females), and 200g can meet that of Mg (260mg/d in males and 220mg/d in females) (FAO, 2002). There are no significant differences between the Kabuli and Desi genotypes except for Ca, with the Desi type having a higher content than the Kabuli type (Ibanez *et al.* 1998; Wang & Daun, 2004). The amounts of total Fe present in chickpea is lower (5.45mg/100g) compared with other pulse crops such as lentils (8.60mg/100g) and beans (7.48mg/100g) (Quinteros *et al*. 2001). Traces reported for Al, Ni, Pb and Cd do not pose any toxicological risk (Quinteros *et al.* 2001). In **Table 6** are reported chickpea mineral content obtained from different studies. Rao & Deosthale (1981) reported mineral content of an Indian chickpea cultivar (growing in Agricultural Univ., Hyderabad, India). Ibanez *et al.* (1998) compared mineral content of Desi and Kabuli type, no discriminable differences are detected.

Minerals	Rao & Deosthale*	Ibanez *et al.*		Wang & Daun				USD
		D	K	D	Range	K	Range	K
Cu	1.18	1.25	1.20	1.00	0.5 – 1.40	1.00	0.7 – 1.40	0.84
Fe	4.60	4.51	4.46	5.90	4.6 – 7.00	5.50	4.3 – 7.60	6.24
Zn	6.11	3.57	3.50	3.60	2.8 – 5.10	4.40	3.6 – 5.60	3.43
Mn	1.21	1.72	1.65	3.40	2.8 – 4.10	3.90	2.3 – 4.80	2.20
Ca	220.0	210.0	154.4	161.7	115 – 226.5	106.60	80.5 – 144.3	105.
Mg	119.0	128.0	122.0	169.10	143.7 – 188.6	177.80	153 – 212.8	115.
Na	-	22.9	21.07	-	-	-	-	24.0
K	-	878.0	926.0	1215.70	1027.6 – 1479	1127.20	816 – 1580	875.
P	398.0	-	-	377.30	276.2 – 518.6	505.1	294 – 828.8	366.
Cr	0.08**	-	-	-	-	-	-	-

K, Kabuli; D, Desi; USDA, United States Department of Agriculture;
*expressed as µg/g;
**the type of chickpea is not specified.

Table 6: Mineral constituents of chickpea seeds (mg/100g) *(*Jukanti *et al.* 2012)

Antioxidants

Important molecules with antioxidant activity present in chickpea seeds are: carotenoids, phenolic compounds (isoflavones, flavonoids, phenolic acids), tocopherols and Vitamin C (Patto *et al*. 2014; Jukanti *et al.* 2012).

The carotenoids present in chickpea seeds include β-carotene, lutein, zeaxanthin, β-cryptoxanthin, lycopene and α-carotene. The average concentration of carotenoids (except lycopene) is higher in wild accessions of chickpea than in cultivated varieties or landraces (cv. *Hadas*) Abbo *et al.* 2005). Two important phenolic compounds found in chickpea are the isoflavones biochanin A (5,7-dihydroxy40-methoxyisoflavone) and formononetin (7-hydroxy-40methoxyisoflavone) (Grusak *et al.* 2007). Other phenolic compounds detected in chickpea oil are daidzein, genistein, metaraminol and secoisolariciresfinol (Champ *et al.* 2000). Sreerama *et al.* (2010) detect the main phenolic compounds in chickpea: ferulic acid, caffeic acid and coumaric acid **(Table 7).** The concentration of biochanin A is higher in Kabuli-type seeds (1420–3080mg/100g) compared with Desi-type seeds (838mg/100g) (Mazur *et al*. 1998).

Legume	Compound	Content	Unit
	Chickpea *(C. Arietinum L.)*		
Cotyledon	Gallic acid	8.5	μg/g d.w.
	Protocatechuic acid	48.0	
	p-Hidroxybenzoic acid	2.1	
	Vanilic acid	57.9	
	Syringic acid	21.9	
	Caffeic acid	103.3	
	Chlorogenic acid	77.4	
	Ferulic acid	159.2	
	Sinapic acid	12.5	
	p-Coumaric acid	99.4	
	Querecetin	7.0	
	Kaempferol	5.5	
	Myrecitin	4.4	
	Daidzein	3.4	
	Geneistein	3.2	

Table 7 Content of individual phenolic compounds in chickpea seeds (Sreerama, Takahashi, & Yamaki, 2012)

According to Torres-Fuentes *et al*. (2011), some peptide fractions obtained from chickpea protein hydrolysates show antioxidant activities, like reducing power, free radical scavenging and cellular antioxidant activity. They are short peptides composed by 8-15 amino acids, obtained from hydrolyzation of legumines, provicilin, phaseolin, vicilin, convicilin, which are common proteins present in chickpea seeds. (Cristina-Torres, Fuentes *et al.* 2014)

Antinutritional factors

Despite the potential nutritional and health-promoting value of anti-nutritional factor (ANF), their presence in chickpea limits its biological value and usage as food. ANF interfere with digestion and make the seed unpalatable when consumed in raw form by monogastric animal species (Domoney *et al.* 1999). ANF can be divided into protein and non-protein ANF. The non-protein ANF include alkaloids, tannins, phytic acid, saponins and phenolics, while the protein ANF include trypsin inhibitors, chymotrypsin inhibitors, lectins and antifungal peptides (Roy *et al.* 2010). Protease inhibitors interfere with digestion by irreversibly binding with trypsin and chymotrypsin in the human digestive tract. They are resistant to the digestive enzyme pepsin and the stomach's acidic pH (Roy *et al.* 2010). ANF can be reduced or eliminated by soaking, cooking, boiling and autoclaving.

1.2.4.4 Physical characteristics

The shape, size and colour of chickpea seeds vary according to different genotypes. As mentioned previously, chickpea seeds are divided in two main market types: i) Desi type is of

Indian origin; their seeds are small, wrinkled with brown, black or green colour, and the bold seeded cultivars of Desi type are often used for roasting. The seeds of Kabuli cultivars (Mediterranean and middle Eastern origin) are large with white to cream coloured seed coat. (Kaur *et al.* 2005).

Cooking time is an important factor used to define cooking quality of pulses. Those that require long cooking times are less convenient, more energy consuming, and, therefore, less desirable for consumers and processors. Pulse breeding programs are interested in breeding for quicker-cooking pulse varieties but, there is no standard method for the evaluation of cooking time. The major direct measures used to evaluate cooking time include sensory analysis, tactile method, spread area ratio, Mattson bean cooker, white core method, and glass slides method. However, each method has limitations, and methodology parameters were found to vary greatly between studies. Cooking time is a heritable characteristic that differs widely among genotypes. One of the main drawbacks that limit the utilization of legumes is their long cooking time (Williams *et al.* 1983). Softening of pulses during cooking is accompanied by structural changes in the seed (Wood *et al.* 2006). Kaur *et al.* (2005) reported main physical changes that occur on chickpea seeds during cooking. Representative samples of six improved commercial chickpea cultivars (4 Desi, 2 Kabuli) from India are represented in **table 8**. Seed weight of different chickpea cultivars varied considerably. Seed weight and volume are positively correlated to fat and carbohydrate content and negatively correlated to fibre content of seeds. The water absorbing capacity of seeds depends on cell wall structure, composition of seed and compactness of the cells in the seed. A positive correlation of swelling capacity with swelling index and hydration capacity was observed. Cooking time for different chickpea cultivars varied significantly. Kabuli cooking time is higher than desi types. It could be related to the rate at which cell separation occurs due to loosening of intercellular matrix of the middle lamella upon cooking. (Kaur *et al.* 2005) Roasting process renders the grain digestible, without loss of nutritious components and, thus, such grains are consumed throughout the world (Sefa-Dedah & Stanley, 1979).

Cultivars	Seed weight (g/100 seeds)	Seed volume (ml/100 seeds)	Seed density (g/ml)	Swelling capacity/seed	Swelling index	Hydration capacity/seed	Hydration index	Cooking tin (min)
Desi type								
PBG-1	12.46 ± 0.44a	9.8 ± 0.31a	1.27 ± 0.03a	0.11 ± 0.01a	108.2 ± 1.98ab	0.12 ± 0.008a	99.2 ± 1.37c	62.4 ± 1.31
PDG-4	16.82 ± 0.66c	12.6 ± 0.50b	1.34 ± 0.03c	0.13 ± 0.009b	103.1 ± 1.66a	0.16 ± 0.01c	92.6 ± 1.01b	78.0 ± 1.65
PDG-3	14.22 ± 0.64b	11.0 ± 0.81ab	1.29 ± 0.03b	0.14 ± 0.01b	127.2 ± 1.42b	0.13 ± 0.009b	91.6 ± 1.09ab	75.0 ± 1.98
GL-769	12.94 ± 0.58a	10.2 ± 0.74a	1.27 ± 0.02a	0.11 ± 0.008s	103.9 ± 1.76a	0.13 ± 0.01b	100.5 ± 1.39d	67.5 ± 1.62
GPF-2	12.91 ± 0.49a	10.0 ± 0.69a	1.29 ± 0.04b	0.12 ± 0.009ab	122.0 ± 1.69ab	0.12 ± 0.009a	96.1 ± 1.14bc	66.0 ± 1.32
Kabuli type								
L-550	21.94 ± 0.52d	17.0 ± 0.82c	1.29 ± 0.04b	0.23 ± 0.01c	136.5 ± 1.39c	0.20 ± 0.01d	90.4 ± 1.23a	95.0 ± 1.52

a Mean ± (standard deviation) for quadruplet measurements. Values with similar letters in a column do not differ significantly ($p < 0.05$).

Table 8: Physicochemical and cooking characteristics of different chickpea cultivars (5 Desi type and 1 Kabuli type). Source: Kaur et al. (2005).

Textural characteristics of legumes may be dependent upon seed microstructure, chemical and physical changes occurring during processing. Soaking and cooking have some effects on seeds texture **(table 9)** (Kaur *et al.* 2005).

Texture profile analysis of soaked seeds of different chickpea cultivars[a]						
Cultivars	**Hardness (N)**	**Cohesiveness**	**Springiness (mm)**	**Gumminess (N)**	**Chewiness (N mm)**	**Fracturablity (N)**
Desi type						
PBG-1	113.3 ± 5.9ab	0.07 ± 0.004ab	3.09 ± 0.05c	8.13 ± 0.31ab	25.2 ± 1.09ab	32.0 ± 1.01b
PDG-4	139.2 ± 4.6c	0.06 ± 0.003a	3.04 ± 0.06c	7.83 ± 0.26a	23.8 ± 0.99a	45.1 ±1.22cd
PDG-3	139.4 ± 6.3c	0.08 ± 0.004b	2.69 ± 0.04ab	11.29 ± 0.30bc	30.4 ± 1.12b	39.4 ± 1.11c
GL-769	106.9 ± 5.5a	0.09 ± 0.007bc	2.89 ± 0.03b	9.15 ± 0.20b	26.5 ± 1.11ab	26.8 ± 1.08a
GPF-2	125.0 ± 3.6b	0.09 ± 0.005c	3.00 ± 0.007c	12.6 ± 0.29c	36.5 ± 1.06bc	29.9 ± 1.17ab
Kabuli type						
L-550	209.7 ± 7.2d	0.08 ± 0.006ab	2.45 ± 0.04a	16.33 ± 0.23d	40.0 ± 1.18c	46.1 ± 1.29d
Texture profile analysis of cooked seeds of different chickpea cultivars[a]						
Desi type						
PBG-1	58.3 ± 2.9c	0.07 ± 0.003a	3.00 ± 0.49d	3.98 ± 0.02b	11.96 ± 0.68d	24.3 ± 1.22d
PDG-4	75.5 ± 3.1d	0.08 ± 0.004ab	1.79 ± 0.24ab	5.67 ± 0.04d	10.20 ± 0.52c	23.2 ± 1.01cd
PDG-3	50.5 ± 2.4b	0.07 ± 0.003ab	2.35 ± 0.39c	3.59 ± 0.02ab	8.43 ± 0.41b	16.9 ± 1.06c
GL-769	43.3 ± 2.3ab	0.12 ± 0.009c	1.95 ± 0.22bc	5.32 ± 0.03cd	10.36 ± 0.56c	12.8 ± 1.09b
GPF-2	39.2 ± 2.1a	0.08 ± 0.002ab	1.55 ± 0.19a	3.09 ± 0.02a	4.79 ± 0.38a	10.5 ± 0.98a
Kabuli type						
L-550	52.2 ± 2.6bc	0.09 ± 0.005b	1.89 ± 0.31b	4.89 ± 0.03c	9.27 ± 0.49bc	15.6 ± 1.09bc

a Mean ± (standard deviation) for octaplate measurements. Values with similar letters in a column do not differ significantly ($p < 0.05$).

Table 9: Textural characteristics of soaked and cooked chickpea seeds (Kaur *et al.* 2005).

1.2.5 HEALTH BENEFITS AND USE IN FOOD INDUSTRY

1.2.5.1 Chickpea in food industry

Chickpeas have been consumed for hundreds of years all over the world, especially in underdeveloped countries where they are an important protein source. Even in the most industrialized countries, the consumption of chickpeas and legumes in general is constantly growing, not only as dried or canned seeds but also as ingredients of functional foods (for example for celiacs, vegetarians) and ready-to-eat products. Chickpea ingredients are not currently recognized as major food allergens by the Food Allergen Labelling and Consumer Protection Act in the United States nor by Regulation 1169/2011 of The European Parliament and the Council of the European Union. (Hernandez *et al*. 2019).

Dried chickpeas can be milled to a flour, which can be added to baked goods to increase their protein content. Chickpea flour can be pressure-cooked in an extruder to make pastas (gluten-free) and puffed snacks. Canned chickpeas can also be roasted to make different snack products. Although cooked chickpeas are not commonly fermented, chickpea-based miso is available commercially. Fermenting chickpeas could enhance their protein digestibility, and used as antioxidant, prebiotic and thickener in bio-yoghurt production. (Hussein *et al.* 2019). In recent years, the discarded water found in canned chickpeas, termed aquafaba, has gained popularity as a plant-based emulsifier in emulsions such as mayonnaise in vegan products (Mustafa *et al.* 2018). Examples of chickpea-derived food products are dried (alone, with other legumes, with cereals), canned (hydrated), flour (pasta, snacks), roasted, hummus, fermented (miso), split (crumbs), and aquafaba. (Hernandez*et al.* 2019)

Processing affects the composition of macronutrients in legumes in various ways. Specific changes to the macronutrient composition may influence how legume ingredients behave in food and during digestion (Daun *et al.* 2006). Processing is used to expand markets and enhance utilization by making legumes more convenient and sometimes nutritionally and functionally equal or superior to other established plant foods on the market. Food processing improves or converts the physical and biochemical properties of relatively bulky, perishable and typically inedible raw materials into more useful, value added, shelf-stable, safe, palatable and economically viable products (Nalle *et al.* 2010; Verma *et al.* 2012). Improvements in digestibility, functionality and suitability of legumes as ingredients in food applications have been gained through modifications of the physicochemical, structural, functional and thermal properties of whole seeds, flours or fractions (Boye *et al*. 2010).

A range of rudimentary/conventional/low-input to advanced/specialized wet and dry treatments, using thermal and non-thermal processing technologies such as milling, cooking/boiling, fermentation, germination, roasting, autoclaving, fractionation (isoelectric precipitation (IEP), ultrafiltration, extrusion, high pressure cooking, and infrared radiation (micronization) has been applied to legume seeds (Arntfield *et al.* 2009).

Common processes applied to chickpea are classified in **Table 10**.

PROCESSING	AIM
Primary processing:	Modify physicochemical charact. (solubility, cooking time, starch content, palatability, digestibility, nutritive value, ANF level, surface area, PSD, water abs., pasting etc.)
- Dehulling;	
- Seed milling;	
Secondary processing:	
Thermal processing (lanching, parboiling, cooking/boiling, autoclaving, roasting, microwave, micronization, canning)	Soaking, germination, fermentation, shelf-life, physicochemical charact., nutritional value, moisture, flavouring, dietary formulations, functional use of isolates
Non-thermal processing (HHP)	
Hydrolysis	
Fractionation	

Table 10: Food processing applied to chickpea and aims. Source: Patto *et al.* 2009

1.2.5.2 Health benefits of chickpea

Although pulses have been consumed for thousands of years for their nutritional qualities (Kerem *et al.* 2007), it is only during the past two to three decades that interest in pulses as food and their potential impact on human health has been revived. Chickpea consumption has been reported to have some physiological benefits that may reduce the risk of chronic diseases and optimise health. Therefore, chickpeas could potentially be considered as a 'functional food' in addition to their accepted role of providing proteins and fibre. Chickpea is a relatively inexpensive source of different vitamins, minerals (Duke *et al.* 1981) and several bioactive compounds (phytates, phenolic compounds, oligosaccharides, etc.) that could aid in potentially lowering the risk of chronic diseases.

CARDIOVASCULAR DISEASE (CVD) – CORONARY HEART DISEASE (CHD) AND CHOLESTEROL CONTROL

Increased consumption of soluble fibre from foods results in reduced serum total cholesterol and Low-density lipoprotein-cholesterol (LDL-C) and has an inverse correlation with CHD mortality (Kushi *et al.* 1999). Chickpea seeds are a relatively cheap source of dietary fibre and bioactive compounds (e.g. phytosterols, saponins and oligosaccharides); coupled with its low glycaemic index (GI), chickpea may be useful for lowering the risk of CVD (Duranti *et al.* 2006). PUFA, LA and OA, constitute almost about 50–60% of chickpea fat. Intake of PUFA such as LA has been shown to have a beneficial effect on serum lipids, insulin sensitivity and haemostatic factors, thereby it could be helpful in lowering the risk of CHD (Hu *et al.* 2001). Moreover, isoflavones may lower the incidence of heart disease due to: the inhibition of LDL-

C oxidation (Tikkanen *et al.* 1998), the inhibition of proliferation of aortic smooth muscle cells (Pan *et al.* 2001) and the maintenance of the physical properties of arterial walls (van der Schouw *et al.* 2002).

DIABETES AND BLOOD PRESSURE

Pulses such as chickpea have a higher amount of resistant starch and amylose. Amylose has a higher degree of polymerisation, rendering the starch in chickpea more resistant to digestion in the small intestine which ultimately results in the lower availability of glucose (O'Dea *et al.* 1992). The lower bioavailability of glucose results in the slower entry of glucose into the bloodstream, thus reducing the demand for insulin which results in the lowering of the GI and insulinemic postprandial response (Osorio, Dıaz *et al.* 2008). The lowering of the GI is an important aspect in reducing both the incidence and the severity of type 2 diabetes (Regina *et al* 2006). LA and b-sitosterol are the major PUFA and phytosterol, respectively, in chickpea seeds; therefore, chickpea seeds could be incorporated as part of a regular diet that may help to reduce blood pressure.

CANCER

Butyrate is a principal short-chain fatty acid (about 18% of the total volatile fatty acids) produced from the consumption of a chickpea diet (200g/d) in healthy adults (Fernando *et al.* 2010). Butyrate has been reported to suppress cell proliferation (Cummings *et al*, 1981) and induce apoptosis (Mathers*et al*. 2002), which may reduce the risk of colorectal cancer. Inclusion of b-sitosterol in a rat diet reduced N-methyl-N-nitrosourea (carcinogen)-induced colonic tumours (Raicht *et al.* 1980). Lycopene, an oxygenated carotenoid present in chickpea seeds, may reduce the risk of prostate cancer (Giovannucci *et al.* 1995). Biochanin A, a chickpea isoflavone, inhibits the growth of stomach cancer cells *in vitro* and reduces tumour growth when the same cells are transferred to mice; further, extract of chickpea isoflavone specifically inhibited epithelial tumour growth and had no effect on healthy cells (Yanagihara *et al*. 1993).

WEIGHT LOSS/OBESITY

Intake of foods, which are rich in dietary fibre (DF), is associated with a lower body mass index (BMI) (Howarth *et al.* 2001). Eating of foods with a high fibre content helps in reaching satiety faster (fullness post-meal), and this satiating effect lasts longer as fibre-rich foods require a longer time to chew and digest in the intestinal system. Additionally, consumption of low-Glycaemic index foods resulted in an increase in cholecystokinin (a gastrointestinal peptide and hunger suppressant) and increased satiety (Holt *et al.* 1992). Chickpea has been

reported to decrease fat accumulation in obese subjects. This aids in improving fat metabolism and could be helpful in correcting obesity-related disorders (Yang *et al.* 2007).

OTHER HEALTH BENEFITS

Chickpea seed oil contains different sterols, tocopherols and tocotrienols. These phytosterols have been reported to exhibit anti-ulcerative, anti-bacterial, anti-fungal, antitumor and anti-inflammatory properties coupled with a lowering effect on cholesterol levels (Arisawa *et al.* 1985). Carotenoids such as lutein and zeaxanthin, the major carotenoids in chickpea seeds, are speculated to play a role in senile or age-related macular degeneration (Arisawa *et al.* 1985). Vitamin A present in chickpea is important in several developmental processes in humans such as bone growth, cell division/differentiation and, most importantly, vision (Arisawa *et al.* 1985).

1.3. PROTEIN EXTRACTION METHODS FROM LEGUME SEEDS

1.3.1. Overview of protein extraction methods

An ideal extraction method for proteins should capture the most comprehensive repertoire of proteins present in the tissue/ matrix considered, while minimizing degradation and contamination by non-proteinaceous compounds and it must be reproducible. Given the diverse biochemical properties of cellular proteins, including their charge, size, hydrophobicity, susceptibility to proteolysis, ligand interactions and subcellular localization, no single protein extraction protocol can capture the full proteome. Comprehensive, uncontaminated and representative protein populations are notoriously difficult to extract from plants, in part because plant cells have a relatively low protein content and are rich in proteases and oxidative enzymes, but mostly because of the metabolic and structural characteristics of plant tissues (e.g. polysaccharides, organic acids, phenolic compounds, terpenes, inhibitory ions, pigments, secondary metabolites and cell wall (Tal Isaacson, 2006). Many techniques have been used for protein extraction and isolation in plant tissues, with subsequent analysis. In case of hard tissues like legume seeds, they must be ground to obtain a fine powder. In this way the cells will be degraded, and protein extraction will be favoured.

General and more specific methods of proteins extraction from the seeds exist. The purpose of the general procedure is to extract proteins from the finely crushed whole seed without considering which specific protein families could be revealed. In this way, total seed storage protein profile can be studied to detect if proteins diversity is present among different

genotypes. Specific protein extractions consist in a set of methods that allow a selective extraction of protein classes like albumins and globulins (e.g. Sodium-phosphate extraction method), amphiphilic proteins (Tris-HCl extraction) and starch granule proteins (Martinant *et al.* 1997).

Regarding protein extraction from plant tissues, the most common protein extraction protocol is based on precipitating proteins from homogenised plant tissue or cells with trichloroacetic acid (TCA) in acetone (Damerval *et al.* 1986), which removes non-protein compounds. TCA/acetone facilitates the dissolution of large amounts of secondary compounds and simultaneously precipitates the proteins. This technique is suitable for various tissues, is simple and aggressively removes interfering compounds, especially lipids and pigments. Limit of this protocol is that proteins are difficult to fully resolubilize.

An alternative protocol is based on the solubilization of proteins in phenol, followed by their precipitation with ammonium acetate in methanol. This method was developed in the 1980s and 1990s (Carpentier *et al.* 2005). In this procedure, proteins are first extracted by using an aqueous buffer, and the resulting extract is then mixed with the buffered phenol (pH 7.8–8.0). This causes the proteins to denature and dissolve into the phenol phase, whereas other water-soluble substances such as salts, nucleic acids and carbohydrates remain in the aqueous phase.

A comparative study (Rose *et al*. 2004) showed that both TCA/acetone and phenol-based methods were effective in extracting and purifying many proteins, but the latter was slightly better in removing interfering compounds from recalcitrant tissues. However, a limitation of many phenol-based protocols is the lack of detergents (e.g. SDS) in the extraction buffer (Hurkman *et al.* 1986). Consequently, the amount of proteins extracted from a sample is often not large enough for global proteomic analysis. In addition, phenol extraction alone is not powerful enough to remove interfering compounds from more complex tissues.

To overcome these problems, Wu *et al.* (2014) designed a novel protein extraction protocol that integrates TCA/acetone precipitation with phenol extraction. This protocol has been shown to have universal applications in the production of high-quality proteins from a wide range of plant materials. An important aspect of this protocol is the use of SDS extraction to link the TCA/acetone precipitation with the phenol extraction. In a classic TCA/acetone protocol, proteins are extracted in rehydration buffer and then directly subjected to protein electrophoretic analysis. However, proteins are extracted by using an SDS-containing buffer. With SDS extraction, the possibility that proteins might not fully resolubilize after TCA/acetone precipitation can be overcome, and the resulting protein extracts can be further

purified by phenol extraction, which has also been modified for use with various plant materials (Wu *et al.* 2014).

Alkaline extraction to solubilize protein (salting-in) from seeds and subsequent acid precipitation of proteins at or near the isoelectric point is the most widely used technique to prepare proteins for food industry (Anson, 1957). An alternative to solubilize protein is the increase of ionic strength by adding salt (salting-out) where proteins precipitate due the less water available to dissolve them. Paredes-Lopez *et al*. (1991) used both the alkaline extraction/acid precipitation procedure and a micellization process (Murray *et al.* 1978) to isolate chickpea proteins; the micellization process involved precipitation of the proteins from a neutral salt extract by dilution in cold water. The micellization has been suggested as a milder approach for the recovery of protein isolate because of less denaturation of the protein structure compared with isoelectric precipitation (Murray *et al.* 1978). Due to the irreversible denaturation of protein isolates, it is not generally recommended to isolate the proteins by carrying out the precipitation procedures such as selective cryoprecipitation, heat coagulation, or addition of metals.

1.3.2. TCA/Acetone extraction method

Precipitating proteins from fined powdered plant tissues with TCA/acetone is a commonly used protein extraction protocol which was developed initially by Damerval *et al*. (1986) to extract wheat seedling proteins. The protocol is based on protein denaturation under acidic and/or hydrophobic conditions that help to concentrate proteins and remove contaminants.

After TCA treatment, at least two washes with Acetone are used to remove residual TCA. During TCA/Acetone precipitation, organic-soluble substances are rinsed out, leaving proteins and other insoluble substances in the precipitate, and proteins are extracted using a buffer of choice. The acidity of TCA (pH <2) causes aggregation and precipitation of all proteins, allowing their separation from the solvent. Different from direct TCA precipitation of protein extracts, TCA/acetone extraction involves the clean-up of tissue powder with 10% TCA/acetone, which is usually more effective than either TCA or acetone alone (Gorg, 1997). Very large volume of plant samples can be handled by TCA/acetone extraction. The success of the TCA/acetone precipitation method is based on the complete pulverization and repeated rinsing of tissue powder to remove the interfering substances (Liangjie Niu, 2018); it is very valuable for inhibition of proteases (Damerval *et al.* 1986) as well as phenoloxidases and peroxidases (Granier, 1988). Plant tissues are rich in proteases, which cause the proteolytic

degradation of proteins and the loss of HMW proteins. However, homogenizing plant tissue in TCA/acetone (-20ºC) almost immediately inactivates proteases and precipitates proteins. Furthermore, TCA/acetone extraction is valuable for removal of interfering compounds and the enrichment of very alkaline proteins such as ribosomal proteins from total cell lysates (Wang, 2008).

However, the resulting pellets from TCA/acetone precipitation may be difficult to dissolve (Wu *et al.* 2014), thus, when this protocol is used, a strong re-solubilizing buffer must be used to ensure efficient re-solubilisation of the precipitated protein and care should be taken not to over-dry the pellet before re-solubilisation. Moreover, prolonged incubation of tissue powder in TCA/acetone may lead to the modification of proteins by acetone, and the proportion of modified peptide might increase over time. Long exposure to the acidic pH in TCA/acetone might cause protein degradation (Wang *et al.* 2006). To overcome these drawbacks, the Laemmli's SDS buffer can be used for protein extraction, because SDS-containing buffers can enhance protein extraction and solubility, especially under heating. SDS (Sodium-dodecyl-sulphate) extraction buffer is a lysis buffer where SDS acts as a detergent to solubilize and denature proteins, separating membrane proteins from membrane because the hydrophobic part of detergent can surround biological membranes and thus isolate proteins. To summarize, the greatest advantages of integrating TCA/Acetone precipitation with SDS extraction are its simplicity and speed (Niu *et al.* 2018). Since it is less time consuming and easier to perform than the phenol-based protocol, TCA/acetone extraction is recommended as a starting protocol for plant protein extraction.

1.3.3. Phenol extraction method

Phenol extraction of proteins is an alternative method to classical TCA/acetone extraction. It allows efficient protein recovery and removes non-protein components in the case of plant tissues rich in polysaccharides, lipids, and phenolic compounds. Phenol-based extraction method was designed by Hurkman and Tanaka (1986), and today is widely used for total protein extraction from plant tissues. Phenol is the simplest aromatic alcohol; it contains a polar $[OH]^-$ group bound to an aromatic ring. It exhibits weak acidic properties and is corrosive and poisonous. Phenol is partially miscible with water: when saturated with water the aqueous layer contains about 7% phenol and the organic layer about 28% water. It interacts with proteins mainly via hydrogen bonding and causes proteins to become denatured and soluble in the organic phase. Then, contrary to widespread belief, proteins are not in the interface but in the phenol phase (Faurobert, 2007).

In this method, proteins are extracted from plant powdered tissues by using a phenol-based solution (tris-buffered phenol) at pH 8.0, followed by methanol (or acetone) precipitation of phenol phase. After phase separation by centrifugation, the phenol phase is "pushed" on top, facilitating to take out it. Phenol dissolves proteins (including membrane proteins) and lipids, leaving water-soluble substances (polysaccharides, nucleic acids, etc.) in the aqueous phase, thus proteins in phenol phase are purified and concentrated simultaneously by subsequent methanol (or acetone) precipitation (Wang *et al.* 2003). Protein pellet from phenol extraction should look white and a yellowish pellet indicates co-precipitation of phenols. Furthermore, when the tissue powder was suspended in the extraction buffer, the addition of 2% w/v SDS to the extraction buffer could improve protein solubility (Wang *et al.* 2003). However, it should be assessed empirically for each specific case.

The success of using phenol extraction depends on keeping samples at low temperature during the first extraction step and carefully recovering the phenolic phase after centrifugation (Faurobert, 2007). Phenol can minimize protein degradation resulting from endogenous proteolytic activity (Schuster *et al.* 1983). Many studies showed that phenol extraction gave satisfactory results in recalcitrant plant tissues rich in components which inhibit electrophoresis (Vincent *et al.* 2006).

Comparisons of TCA/acetone and phenol extraction protocols done by Carpentier *et al*. (2005) and Saravanan and Rose (2004) indicated that both methods are efficient in extracting proteins from recalcitrant tissues, but phenol extraction is most efficient in removing interfering substances and resulted in the highest quality gels with less background and less vertical streaking. The two methods minimize the protein degradation often encountered during sample preparation, owing to endogenous proteolytic activity. It was also pointed out that the phenol method yielded a greater number of glycoproteins (Saravanan and Rose, 2004). The phenol extraction procedure has a high clean-up capacity. Phenol also acts as a dissociating agent decreasing molecular interaction between proteins and other materials (Carpentie, 2005). The major drawbacks of the protocol are that it is time consuming (at least 6 hours) and that phenol and methanol are toxic.

1.3.4. Other protein extraction methods for plant tissue from literature

Along with TCA/Acetone and Phenol methods, other protein extraction approaches were proposed for legume seeds in literature. Alli *et al*. (1980) have been proposed two protein extraction methods based on citric acid and sodium hydroxide (NaOH) solutions. Procedures

were applied to extract proteins from *Phaseolus* beans. Alkaline extraction (fine powdered tissue mixed with NaOH solution) is generally followed by acid precipitation (proteins precipitate at or near isoelectric point). Citric acid protein extraction consists of mixing citric acid solution with sample flour. This process is used to extract soluble proteins and generally is followed by lyophilization of isolates. (Melnychyn, 1969).

Granier *et al*. (1988) described extraction of soluble proteins with a non-denaturing Tris-buffer. Dry powder is suspended in 2 mL pre-chilled extraction buffer containing 30mM of Tris-HCl, pH 8.7. Since the Tris procedure allows only the recovery of soluble proteins, membrane-bound proteins will be absent. TCA/Acetone extraction allows to detect a higher number of protein classes than Tris-buffer extraction in the 2D-PAGE analysis.

Natarajan *et al.* (Natarajan *et al.*2005) compared urea, thiourea/urea, TCA/Acetone and phenol protein extraction methods to determine their efficacy in separating soybean seed proteins by 2D-PAGE. In urea extraction method, soybean seeds were ground to fine powder and proteins were extracted by lysis buffer containing 8M Urea. Urea/thiourea extraction buffer contains 5M urea and 2M thiourea. Among the four methods, urea solubilization and phenol extraction resolved fewer proteins spots than thiourea/urea and TCA/Acetone extraction method.

Other buffer systems could be used for seed proteins extraction. Hameed *et al*. (2009) and Aarif *et al*. (2015) described total protein extraction from chickpea fine powdered tissue with 50mM sodium phosphate buffer (pH 7.6) followed by SDS-PAGE analysis.

CHAPTER II: MATERIALS AND METHODS

2.1 MATERIALS

Six different genotypes of chickpea have been analysed in this study **(table 11)** to set up the protocol to efficiently extract proteins from chickpea seeds and to preliminary investigate if differences in seed protein content and protein profile exist within this small sample. The different genotypes belong to a wide collection of about 1,900 different chickpea genotypes developed by the Plant Genetic research group of Department of Agricultural, Food and Environmental Sciences of Polytechnic University of Marche. Samples of the whole chickpea collection were mainly from two gene banks, Leibniz Institute of Plant Genetics and Crop Plant Research-IPK (Gaterselben, Germany) and United States Department of Agriculture (USDA) Western Regional Plant Introduction Station (USA).

Code	Common name/ synonym code	Country of origin	Biological status	Market type	Seed colour
AN_Ca_2117	Palazzo S. Gervaso	Italy	Landrace	Kabuli	White
AN_Ca_2118	Nero Tolve	Italy	Landrace	Desi	Black
AN_Ca_2110	Pascià	Italy	Cultivar	Kabuli	White
AN_Ca_2114	Ituchi	Spain	Cultivar	Kabuli	White
AN_Ca_0009	CIC63	Bulgary	Breeding material	Desi	Red
AN_Ca_0097	CIC137	Italy	Landrace	Desi	Brown

Table 11: List of chickpea genotypes used in this study.

The following buffers and solutions were used in TCA/Acetone extraction method:

- TCA/Acetone buffer (10% TCA (w/v) in acetone and freshly added 10mM DTT), stored at - 20ºC;
- Acetone solution (80% acetone (v/v) in water and freshly added 10mM DTT), stored at − 20 °C;

- SDS extraction buffer (1% SDS, 0.15M Tris/HCl, 1mM EDTA, and freshly added 0.1M DTT, 2mM PSMF), stored at 4 °C.

The following buffers and solutions were used in Phenol extraction method:

- Tris-buffered phenol solution (stored at 4 °C, pH 7.8–8.0; Sigma-Aldrich);
- Ammonium acetate solutions (0.1M Ammonium acetate in pure MeOH), stored at −20°C;
- Washing buffer (10mM Tris-HCl, 1mM EDTA, 0.7M sucrose), stored at 4 °C;
- SDS-free extraction buffer (0.15M Tris-HCl, 1mM EDTA and freshly added 0.1M DTT, 2mM PSMF), stored at 4 °C.

After extraction, the proteins were quantified by carrying out the Bradford assay (Bradford, 1976) with the aid of the spectrophotometer Beckman coulter DU800.

The qualitative protein analysis was carried out by SDS-PAGE analysis in accordance with Laemmli (1970).

The electrophoresis chamber and handcasting equipment (slide and comb 1 mm) were obtained by Biorad®. Compositions and solutions used in the electrophoresis analysis are as follows:

- Polyacrylamide running gel 12% (62mM Tris/HCl pH 6.8; 0.1% w/v SDS; 11.7% w/v Acrylamide; 0.3% w/v Bis-acrylamide; 0.2% w/v APS; 0.05% w/v Temed);
- Polyacrylamide running gel 15% (62mM Tris/HCl pH 6.8; 0.1% w/v SDS; 14.6% w/v Acrylamide; 0.3% w/v Bis-acrylamide; 0.2% w/v APS; 0.05% w/v Temed);
- Polyacrylamide stacking gel 3% (50mM Tris/HCl pH 6.8; 0.1% w/v SDS; 2.92% w/v Acrylamide; 0.3% w/v Bis-acrylamide; 0.2% w/v APS; 0.05% w/v Temed);
- Running buffer used to fill electrophoretic chambers was composed of 25mM Tris, 0.192M Glycine and 0.1% SDS;
- SDS sample buffer 4x (40% w/v glycerol; 248 mM Tris/HCl pH 6.8; 20% v/v β-Mercaptoethanol; 0.004% w/v bromophenol blue; 8% w/v SDS) has been used for preparation of electrophoresis samples;

- Unstained protein MW marker (Pierce™) composed of a mixture of seven purified proteins ranging from 14.4 kDa to 116 kDa has been used in all analysed SDS-PAGE gels;
- Coomassie blue staining buffer (0.1% w/v Brilliant blue Coomassie R250; 10% v/v Acetic Acid; 50% v/v methanol) has been used to colouring the polyacrylamide gel;
- Strong de-staining buffer (10% Acetic ac. and 50% methanol) and weak de-staining buffer (7% Acetic ac. and 5% methanol) have been used to remove excess dye;
- The electrophoresis of six different chickpea genotypes has been carried out by using a precast AnyKD Mini-PROTEAN TGX Stain-Free Protein Gel for separation of polypeptides of 10–200 kDa (Biorad®).

2.2 METHODS

2.2.1 Sample preparation

The first step was to compare two different methods for grinding the chickpea seeds to obtain fine powder. One method was to use mortars and pestles under liquid nitrogen, while the other was to grind tissues by the use of TissueLyser (Qiagen®), a small bead mill which provides fast, effective disruption of up to 192 samples at the same time. Seeds were put in 2 ml tubes with a tungsten bead for each sample; the grinding occurred at low temperature by the use of plates (adapters for plastic tubes) build to be cooled in liquid nitrogen and to maintain the low temperatures during the shaking, which was performed at high speed (8 min, 30 Hz). Powders obtained were stored at -80º C.

2.1.1 TCA/Acetone extraction method

TCA/Acetone extraction method is based on the protocol described by Wu *et al.* (Xiaolin Wu, 2014). An aliquot of seed powder is transferred to appropriate plastic tube making sure not to thaw the powder, then the seed powder was weighed and 10 ml of cold (-20°C) TCA/Acetone is added per 0.5g of seed powder according to the following proportion:

$$0.5g : 10ml = powder\ weight : x \quad (1)$$

The sample was centrifuged for 8 min at 11,000g, at 4ºC. The supernatant is discarded, and the operation is repeated two more times to obtain a white or whitish precipitate. The final pellet was suspended in cold (-20°C) acetone (the volume is calculated according to equation

1) and the solution was centrifuged at 11,000g for 8 min at 4ºC. The supernatant is discarded, and the step was performed two more times. The pellet was left about 30 minutes under the chemical hood in order to eliminate any acetone residue. The pellet was subsequently resuspended with 1.5ml of SDS-extraction buffer for 0.1g of weighed seed powder following this proportion:

$$0.1g : 1.5ml = pellet\ weight : x \quad (2)$$

The sample was incubated at 65ºC in a water-bath for 2h in order to favour protein resuspension. Before incubation, a Protease inhibitor cocktail is added to inhibit protease activity. The sample is centrifuged at 11,000g for 15 min at room temperature. Finally, the supernatant containing the protein extract is divided into 1ml-aliquots and stored at -20ºC if not immediately analysed.

2.1.2 Phenol extraction method

Phenol extraction method used in this study is based on the protocol described by Wu *et al*. (Xiaolin Wu, 2014). Part of the protein extract obtained with TCA/Acetone extraction method was processed as follows; an equal volume of Tris-buffered phenol was added, and the solution was stirred to obtain an emulsion, that was centrifuged at 11,000g for 8 min at room temperature in order to obtain a separation of phases. The phenolic phase was collected, and an equal volume of washing buffer is added. After mixing, the solution was centrifuged at 11,000g for 8 min at room temperature. Then 1.5ml of ammonium acetate solution was added to 200µl of sample following this proportion:

$$1.5ml : 200\mu l\ (sample) = x : sample\ volume \quad (3)$$

The solution was mixed and incubated at -20ºC for at least 30 min. After centrifugation at 11,000g for 8 min at 4ºC, proteins were present in the pellet, while phenol was dissolved in methanol. The pellet was collected, and an equal volume of Ammonium acetate solution was added, after centrifugation as above; the pellet was washed with Acetone solution. After centrifugation, the supernatant was removed, and the pellet is dried under the chemical hood to remove the acetone. The pellet can be immediately analysed or preserved at -20ºC.

2.1.3 Protein analysis

Spectrophotometric analysis has been carried out in order to analyse the protein concentration of the seeds of the six chickpea genotypes by Bradford assay (M.Bradford, 1976). Bovine

serum albumin (BSA) has been used as standard reference for the quantification of protein content. SDS-PAGE has been performed following Laemmli method (Laemmli, 1970).

Four different SDS-PAGE analyses have been conducted:

1) The protein profile resulting from the two different grinding methods of chickpea seeds was compared. To do it the same genotype was used for protein extraction. Six samples of chickpea powder of AN_Ca_2117 (*Palazzo San Gervasio*) genotype have been analysed. Three of them (**Figure 3**: 1.1, 1.2, 1.3) were grinded with mortar and pestle, while the remaining samples (**Figure 3**: 5.1, 5.2, 5.3) were grinded with the Tissue Lyser. TCA/Acetone extraction method has been used to extract proteins. The samples for SDS-PAGE contained 30µg of protein and were boiled at 100°C for 5min. Gels were ran at 10mA for 2 hours. The gel was, then, stained with Coomassie blue dye overnight with agitation and subsequently destained with the above reported solutions.

2) The six samples of chickpea of AN_Ca_2117 (*Palazzo San Gervasio*) genotype have been analysed by SDS-PAGE in order to compare the protein profile between TCA/Acetone and Phenol extraction. For the electrophoretic analysis, three TCA samples and three PHE samples have been prepared with a final protein amount of 10, 20, and 50 µg. All samples were boiled at 100ºC for 5min. Electrophoresis was performed at 10mA for 2 hours. Gel was, then, stained with Coomassie blue dye overnight with agitation and subsequently destained with the above reported solutions.

3) Three chickpea genotypes, AN_Ca_2118 (*Nero Tolve*), AN_Ca_2117 (*Palazzo San Gervasio*) and AN_Ca_0009, were analysed by loading into the gel different amounts of proteins to determine the appropriate protein quantity that allows the best visualization of the protein profile. TCA/Acetone extraction method has been used. Three samples for each genotype with a final protein quantity of 30, 60 and 90µg have been prepared. Hand cast-polyacrylamide gels were used. All samples were boiled at 100º for 5min. Electrophoresis was performed at 10mA for 2 hours. A second electrophoretic analysis was performed with precast polyacrylamide gels (precast AnyKD gel from Biorad®) to analyse the protein profile resolution of four samples of Palazzo San Gervasio local variety with different protein content (less than 30 µg). Electrophoresis was performed at 150V at 4ºC. Gels were, then, stained with Coomassie blue dye overnight with agitation and subsequently destained with above reported solutions.

4) The same amount of proteins (15µg) extracted from the seeds of all the six chickpea genotypes considered **(Table 10)** were compared after electrophoresis. TCA/Acetone extraction method has been used to extract the proteins. The samples for SDS-PAGE contained 15µg of proteins and were boiled at 100°C for 5min. Samples were loaded on the precast polyacrylamide gel. Gel was, then, stained with Coomassie blue dye overnight with agitation and subsequently destained with the above reported solutions.

CHAPTER III: RESULTS

3.1 Effects of different grinding procedures on the protein profile

Six samples of chickpea powder have been obtained from seeds of AN_Ca_2117 (*Palazzo San Gervasio*) genotype by applying two different grinding methods as previously described. **Figure 3** shows the protein profile of the samples after loading onto a 12% polyacrylamide gel; in particular, samples 1.1, 1.2, 1.3 were those grinded with mortars and pestles under liquid nitrogen, while the samples 5.1, 5.2 and 5.3 were grinded with the TissueLyser, at low temperature guaranteed by plates (adaptors for plastic tubes) cooled with liquid nitrogen. The aim of this experiment was to investigate if differences exist among these two procedures and to identify the most efficient method in term of rapidity and efficacy to obtain a fine powdered tissue as starting material. Indeed, having a fine powdered tissue is needed to start protein analysis of plant materials (Wu *et al.* 2014). The results of this experiment are really very important, especially if the goal is to analyse seeds of a large collection of genotypes, in order to optimize the time required for the preparation of the samples. In both cases, liquid N_2 is essential to minimize proteolysis and protein modification that may occur during tissue disruption.

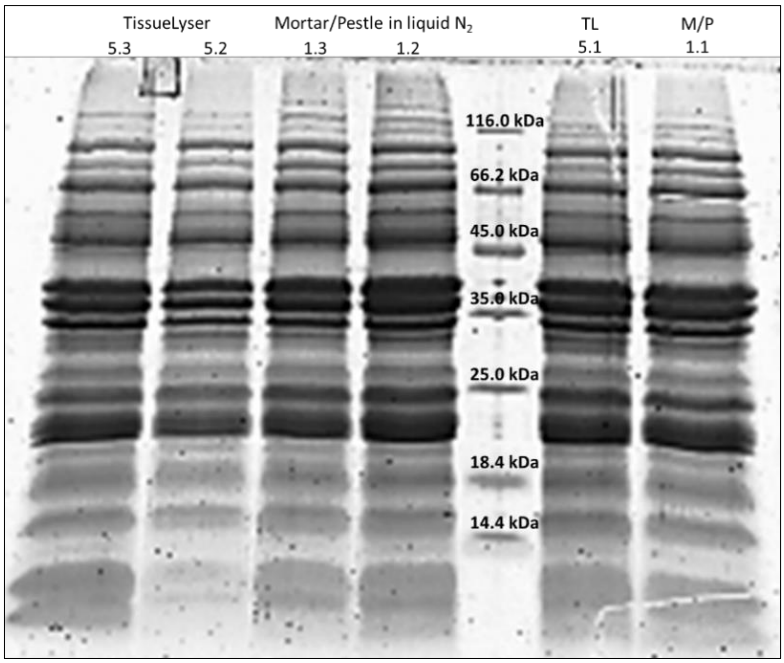

Figure 3: 12% polyacrlylamide gel obtained from SDS-PAGE of six samples of Palazzo San Gervasio chickpea genotype. Proteins have been extracted by TCA/Acetone extraction method. Protein amount

of samples is 30μg each one. Samples 1.1, 1.2 and 1.3 have been prepared by mortar and pestle (M/P9; 5.1, 5.2 and 5.3 have been prepared by TissueLyser (TL).

As shown in **figure 3**, loading 30μg of total protein for each sample allowed to discriminate a wide range of protein classes. The most abundant proteins can be observed in the range of 20-25 kDa and 35-45 kDa. A noticeable protein concentration is present in the bands with MW 66.2 kDa and 116 kDa. Samples prepared with mortar and pestle show a better discrimination of HMW bands (higher than 116 kDa), while discrimination of LMW bands is similar, except for sample 5.2 (TissueLyser) where bands less than 14 kDa were lost. The similarity of the profiles indicates that the two methods are very similar in terms of effectiveness, that is, no loss of proteins occurred with the two methods. The powder obtained with TissueLyser has shown a greyish colour, probably due to a contamination with the tungsten beads that are used during the grinding process to favour a greater fineness of the powder. However, this anomaly does not appear to have affected the results. The procedure with TissueLyser, however, required more time and resulted less convenient for the preparation of the samples, as it still requires a preliminary disruption of the seeds with mortar and pestle.

3.2 Effects of different extraction procedures on the protein profile

Six samples of chickpea powder have been obtained from seeds of AN_Ca_2117 (Palazzo San Gervasio) genotype and analysed by SDS-PAGE with the aim being the comparison of the protein profile of samples extracted with TCA/Acetone method and Phenol method in order to detect the best extraction method that could be used for chickpea proteomic analysis.

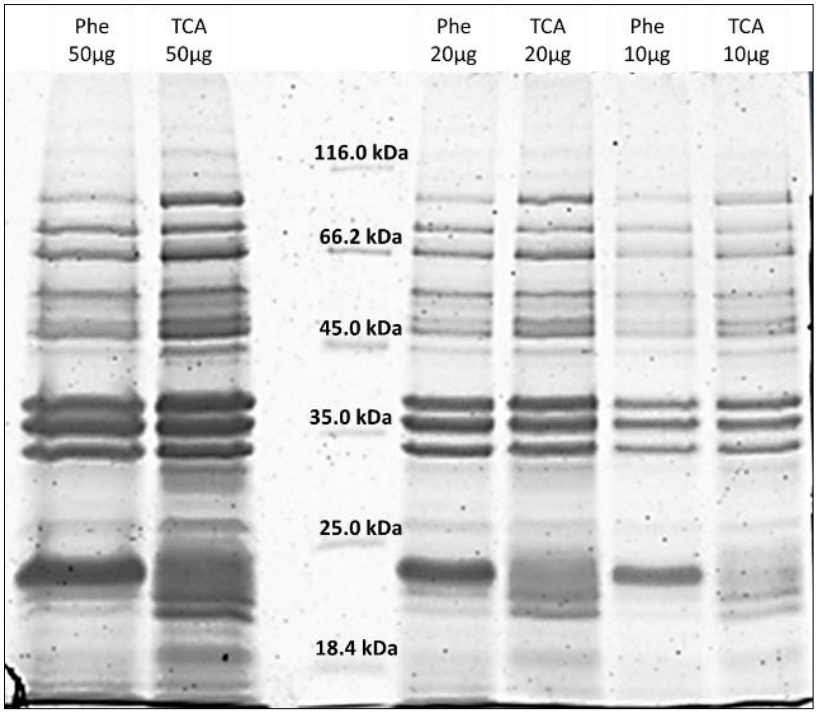

Figure 4: 15% polyacrylamide gel obtained from SDS-PAGE of three protein extracts obtained with TCA/Acetone method (TCA) and three obtained with Phenol extraction method (Phe). Samples of 30µl were prepared with a different protein amount, respectively 10, 20, and 50 µg.

Figure 4 shows a 15% polyacrylamide gel from SDS-PAGE of three protein extracts obtained with TCA/Acetone method (TCA) and three obtained with Phenol extraction method (Phe), loaded into the gel at different protein contents (10, 20 and 50 µg), in order to have a better discrimination between bands. A band of higher intensity (22 kDa) was observed in all the PHE samples compared to TCA samples; however, more LMW bands (< 22 kDa) were present in TCA samples compared to PHE samples. Bands around 30-40 kDa (which represent protein subunits present with higher concentration in chickpea seeds), are well discriminated in both in TCA and PHE samples. Bands around 45-60 kDa are slightly more pronounced in TCA samples, while HMW bands are similar in both TCA and PHE samples. At a protein concentration of 50µg, a higher protein concentration of high-MW bands is present in TCA sample compared to PHE sample. According to Ramu *et al.* (2004), Phenol extraction method statistically give a better yield in LMW-proteins extraction for proteomics of plant tissues. However, as shown in **Figure 4**, no significant differences are observed in the samples extracted with the two methods, except for the band around 25 kDa, which results more

concentrated in PHE samples. Regarding HMW bands, some of them are more visible in TCA samples, except for 10µg samples.

TCA extraction resulted much faster and safer than PHE extraction method, as phenol is a highly toxic and corrosive compound. Moreover, the phenol extraction method has required much more time and a greater complexity of operations.

3.3 Comparative protein profile from different chickpea genotypes

3.3.1 Comparative protein profile of three chickpea genotypes

In **Figure 5**, protein complex profile of three chickpea genotypes, GER (AN_Ca_2117, *Palazzo San Gervasio*), Nero (AN_Ca_2118, *Nero di Tolve*) and AN009 (AN_Ca_0009) have been compared by SDS-PAGE. Three samples for each genotype have been prepared with an increasing amount of proteins (30, 60 and 90 µg) determined by quantitative protein analysis with Bradford assay.

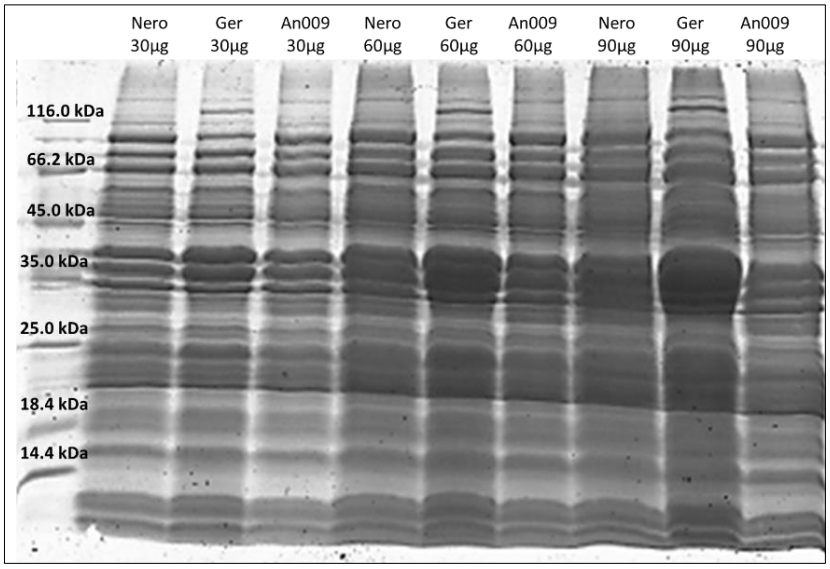

Figure 5: 15% polyacrylamide gel from SDS-PAGE of protein extracts obtained with TCA/Acetone method. Three genotypes (Nero=Nero di Tolve; Ger=Palazzo San Gervasio; An009=AN_Ca_009) have been analysed. For each genotype, three samples with different protein amounts (30, 60, 90 µg) have been loaded.

The aim of this experiment was to identify the protein amount that allows to obtain the best discrimination of LMW and HMW bands. As shown in **Figure 5**, in all samples, the bands ranging from 14.4 kDa to 116.0 kDa were detected. However, the best discrimination was

observed when the three samples had a protein amount of 30µg. With this amount, bands with MW higher than 116 kDa are not detectable, but the bands between 20 and 60 kDa, that represents the protein classes most concentrated in chickpea seeds (Sanchez-Vioque *et al.* 1999), are well visible and separated. Samples with protein amount of 60 and 90 µg allows a higher discrimination of HMW bands, but they show an overlap of bands in the range 20-45 kDa for 60µg and 25-60 kDa for 90 µg, LMW bands are well visible in all samples.

Another important outcome of such experiment was the reproducibility of the samples, indeed the samples representing the same genotype showed an increase in the intensity of the bands of the same MW directly proportional to the increase in the amount of proteins. Moreover, different genotypes show a similar protein content, based on the distribution of the various bands on the gel. The genotype AN_Ca_2117 (*Palazzo San Gervasio*) showed a higher content of proteins in the range 35-45 kDa than the other genotypes, and this is evident in all samples with different protein amount.

3.3.2 Comparative protein profile of San Gervasio genotype with different protein amounts

This experiment has been carried out to compare the resolution of protein classes in chickpea genotype AN_Ca_2117 (*Palazzo San Gervasio*) samples with protein content ranging from 7.5 to 30µg. As shown in **Figure 6**, for samples at 3.3µg and 7.5µg protein content, the bands are too faint for further analysis. The sample at 15µg protein content shows a wide range of clear bands, and a better discrimination of 20-25 kDa and 35-45 kDa proteins, than the sample at 30µg. This experiment helped us to define the protein content that should be used to compare the six chickpea genotypes.

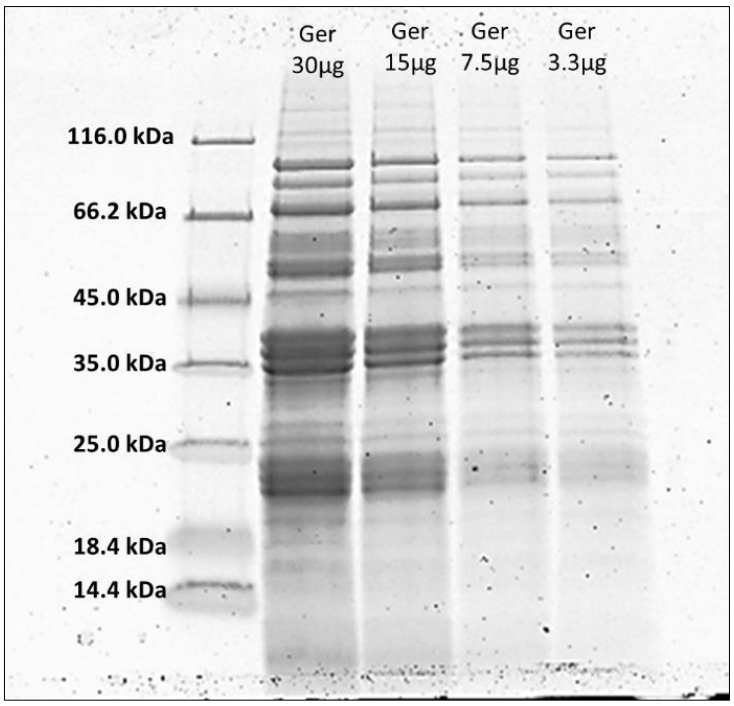

Figure 6: Precast AnyKD polyacrylamide gel (Biorad®) of four samples of Palazzo San Gervasio (Ger) chickpea genotype with different protein amount (3.3 to 30 µg). All samples were extracted with TCA/Acetone method.

3.3.3 SDS-PAGE gel: comparative protein profile of six chickpea genotypes

Protein complex profile of six chickpea genotypes **(Table 10)** have been compared by SDS-PAGE analysis in order to detect differences in protein content among genotypes of the same species (C. *arietinum L.*). The results are shown in **Figure 8**. All samples were extracted by TCA/Acetone method. In **Figure 7** are shown the protein extracts of chickpea genotypes obtained from TCA extraction method that have been used for this experiment. Quantitative protein analysis expressed as mg/g of dry flour has been performed by Bradford assay.

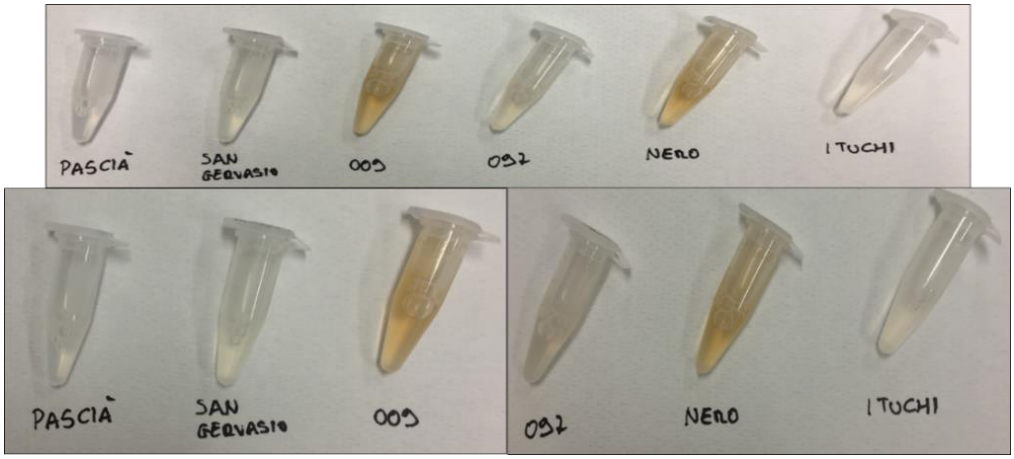

Figure 7: SDS-protein extracts of six chickpea genotypes obtained by TCA/Acetone method. Nero (Nero di Tolve) and AN_Ca_009 (009) brownish colour could be related to the presence of some pigment residues, typical of Desi cultivars, that present seeds from green-brown to black colour.

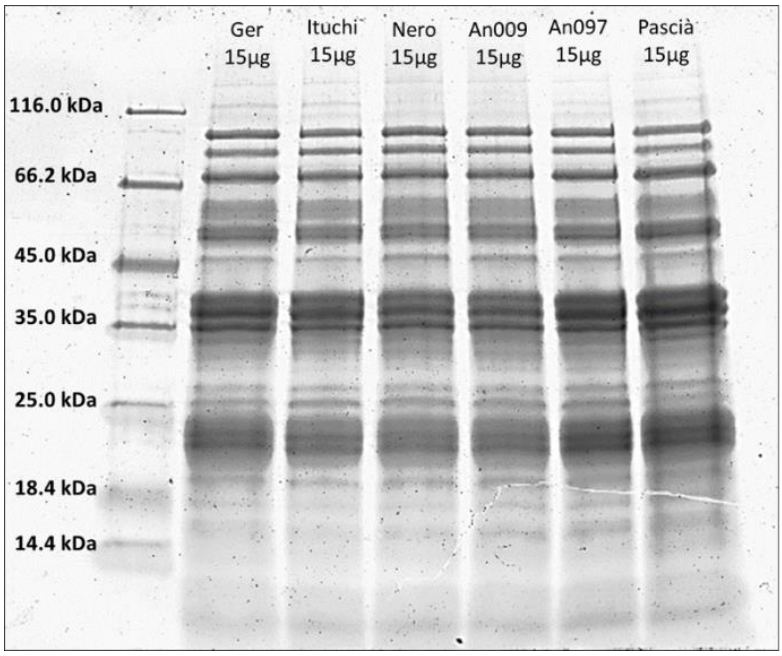

Figure 8: SDS-PAGE Polyacrylamide gel (precast AnyKD Mini-PROTEAN TGX Stain-Free Protein Gel, Biorad®) of six chickpea genotypes: Palazzo San Gervasio (GER), Ituchi, Nero di Tolve (Nero), AN_Ca_009 (AN009, AN_Ca_097 (AN097) and Pascià. Samples of 30µl have been loaded on the gel, with a protein content of 15µg.

In all samples the most abundant proteins are in the range of 20-25 kDa and 30-40 kDa, in accordance with the results of the previous experiments. Samples Nero (AN_Ca_2118, Nero Tolve) and AN009 (AN_Ca_0009) showed a less protein concentration at range 20-25 kDa

than other samples. Although LMW bands have a less resolution than others, it is possible to observe higher concentration of proteins around 15 kDa in AN_Ca_0097 and Pascià genotypes. HMW bands have a good resolution.

The total protein content in the seeds of the different chickpea genotypes is reported in **Table 12.**

Code	Common name/ synonym code	Protein content (mg/ g dry flour)
AN_Ca_2117	Palazzo S. Gervaso	59.09
AN_Ca_2118	Nero Tolve	65.30
AN_Ca_2110	Pascià	12.71
AN_Ca_2114	Ituchi	50.53
AN_Ca_0009	CIC63	62.58
AN_Ca_0097	CIC137	60.67

Table 12. Protein content of the six different chickpea genotypes analysed by Bradford assay, expressed in mg/g of dry flour.

CHAPTER IV: DISCUSSION AND CONCLUSION

4.1 OPTIMIZATION OF PROTEIN EXTRACTION METHOD AND PROTEIN ANALYSIS

The main aim of this work was to develop a protocol to efficiently determine the total protein content of seeds from large collections of chickpea genotypes. It will represent a very useful tool to phenotype different chickpea genotypes (i.e. plant genetic resources) for this seed quality trait.

Considering the fact that the procedure has to be applied to numerous samples, it should be rapid and efficient, allowing to optimize the time for extraction and analysis of the total protein content, without compromising the quality of the seed (i.e. trying to preserve the entire proteome as much as possible).

Common procedure before protein analysis is to grind seeds into a fine powdered tissue to disrupt cell wall and release proteins (Wu *et al*, 2014). In this study we applied two different methods to grind the seeds, both at low temperature by using liquid nitrogen. Among the use of a mortar and pestle and that of a TyssueLyser machine, the former resulted faster to obtain a fine and homogeneous powder than the latter, especially for the fact that the procedure with TissueLyser requires a first step of disruption of seeds with mortar and pestle, because alone it cannot grind the entire seed. Concerning quality and quantity of proteins extracted the two methods gave very similar results. If the aim is to have an efficient method, the mortar and pestle method remains the best procedure considering that is faster than the use of a bead mill.

Most published proteomics studies of plant tissues use a procedure in which proteins are precipitated with TCA and acetone, followed by solubilization in a detergent (e.g. SDS). According to Ramu *et al.* (2004), this approach increases protein concentration and helps remove contaminants, although some polymeric contaminants could be coextracted. An alternative protocol has been developed in which proteins are solubilized in phenol (phe) and subsequently precipitated with methanol and ammonium acetate, followed by resolubilization. In literature there is discordance between the choice of the most efficient method for the extraction of proteins from plant materials: some studies suggest that extraction with phenol allows to obtain purer extracts (i.e. with minimal contamination from other components) compared to extraction with TCA (Natajan *et al*, 2005), while others suggest that there are no

statistically significant differences in the protein yield among the two protocols (Maldonado *et al*, 2008).

We compared these methods to extract proteins from chickpea powdered tissues, to detect the most efficient one. TCA extraction method reduces time and materials required for protein extraction compared to Phenol extraction. Even if literature sometimes suggests the use of Phenol for protein extraction, according to our results (**Figure. 4**) TCA method not only discriminated better low and high molecular weight bands, but also more intense bands were obtained (therefore composed of a greater number of proteins) compared to extraction with phenol. This result suggests that the extraction with TCA in Acetone, with the use of Sodium-dodecyl sulphate (SDS) as detergent for solubilization, applied to powdered chickpea seeds can be advantageous in terms of efficacy and efficiency.

SDS has been used to solubilize proteins and obtain samples that can be analysed by SDS-PAGE. SDS breaks non-covalent bonds in proteins and therefore denatures them, causing the loss of their native conformation. In addition, the anionic component of the SDS binds the peptide chain; this confers a negative charge to the protein proportional to its mass (about 1.4 g SDS / g protein). This negative charge is significantly greater than the original electric charge. The electrostatic repulsion that is created by the binding of the SDS causes the denaturation of the protein to a filiform structure, eliminating the migration differences due to the difference in structure. This allows to separate proteins exclusively based on their molecular weight.

Different experiments have been carried out for SDS-PAGE analysis, with variation in polyacrylamide gel composition and µg of samples loaded. 12% and 15% polyacrylamide gels are similar in term of resolution; however, 12% gel gives a better resolution of HMW bands and LMW bands can be lost, vice versa for 15% gel, which gives a better resolution of LMW bands. Regarding the use of Any kD precast gel (Biorad®), the main advantage is that time is not wasted for gel preparation (approximately 1 hour) and electrophoretic run (saving about 30 min), and it gives a better resolution of bands higher than 25 kDa, while smaller bands are lost, as shown in **Figure 6** and **Figure 7**. We found that 15% gel gives the best resolution in a wide range of molecular weights, however, we are interested principally to MW higher than 25kDa, which correspond to proteins subunits particularly abundant in chickpea. Considering the advantages and reproducibility of precast gel, we suggest its use to identify different protein profiles among diverse genotypes.

Another comparison made during experiments was between different protein amounts loaded on gels. This is a critical aspect that must be considered to optimize protein analysis, since an excessive quantity allows to observe a greater number of bands, but with the risk of having an overlap of thebands corresponding to the most abundant proteins. In **Figure 5** as example, it is possible to observe that a protein content of 90µg causes an overlap of bands. Vice versa, a low amount of protein causes the loss of many bands, especially those less concentrated. According to our experiments, loading protein amounts less than 20µg can cause loss of many bands when using handmade polyacrylamide gels, while loading an amount of 15µg is optimal for resolution when using the precast gel .

To summarize, our study allowed to define an optimal protocol to extract proteins from chickpea seeds, and to look at differences in protein profiles of diverse chickpea genotypes; here the most important steps:

1. To grind the chickpea seeds by mortar and pestle with liquid nitrogen;
2. TCA/Acetone extraction method;
3. Solubilization in SDS;
4. Bradford assay;
5. SDS-PAGE: Any kD precast gel (Biorad®), loading samples with protein content of 15µg;
6. Coomassie blue staining.

4.2 DISCRIMINATION OF PROTEIN CONTENT OF SIX CHICKPEA GENOTYPES

Seeds of six chickpea genotypes, including cultivars and local varieties, have been analysed to have a preliminary comparison of protein content and profiles. Four genotypes (AN_Ca_2117, AN_CA_2110, AN_Ca_2114, AN_Ca_0097) are Kabuli type, while two (AN_Ca_2118 and AN_Ca_0009) are Desi type. Even if samples derived from proper replicated experiments are needed to identify significant differences in protein content of the different genotypes, this study allowed to highlight variation in the protein content of the six genotypes considered, with a range from 50.5 to 65.3 mg/g of dry flour for AN_Ca_2114 (Ituchi cultivar) and AN_Ca_2118 (Nero Tolve landrace), respectively; protein content of AN_Ca_2110 genotype was not considered because it was very low, 12.71 mg/g of dry flour,

thus this sample has to be repeated to confirm such low value. Moreover, Desi types showed a higher content of protein compared to Kabuli types.

Interestingly, along with diversity in total protein content among the diverse genotypes, also differences in protein profiles were observed. AN_Ca_2117 (Palazzo San Gervasio) genotype showed a higher protein content than AN_Ca_2118 (Nero Tolve) and AN_Ca_0009 genotypes in the range 20-25 and 30-45 kDa (**Figure 5** and **Figure 7**). Kabuli genotypes showed a higher protein content on this range than Desi cultivars.

The higher protein concentration present in all samples is on the range 20-25 kDa, 35-40 kDa and 50-70 kDa. According to Vioque *et al.* (1999), the range 20-25 kDa corresponds to β-legumines (25.3 to 24.3 kDa), vicilin (19 kDa) and albumins (24-26 kDa). The range 30-45 kDa corresponds to α-legumines (46.5 to 39.8 kDa), vicilin (33-37 kDa) and albumins (35 kDa). Similar values are reported also by Yu-Wei (2006), Gueguen (1991) and Vairhinos *et al* (1982), where 40, 39, 23 and 22 kDa correspond to 11S-globulin (legumin), 50, 37-33, 19-15 kDa correspond to 7S-globulin (vicilin). This comparison confirms that globulins (11S and 7S subunits) and albumins are the most abundant protein classes in chickpea seeds, in accordance with data reported by Singh and Jambunathan (1982). Globulins and albumins are associated also to other molecular weights. Globulins are present at 73, 62 and 60 kDa, while albumins in 110 and 54 (2S subunit) (Vairinhos and Murray, 1982). Minor protein classes present in chickpea are glutenins. This protein corresponds to molecular weights like 110 kDa (Chavan *et al*, 1989) and 55 kDa (Takaiwa *et al.* 1999). Subunits of MW 96 kDa could be the chickpea lipoxygenase (92 kDa) reported by Clemente *et al*. (2000); similar MW of lipoxygenase (93.3 kDa) reported by Sathe *et al*. (1987) was observed in soybean isolates.

Differences in intensity of the bands of diverse molecular weight indicate that differences exists among the different genotypes; moreover, presence/ absence of a protein band at 116 kDa was also observed, with presence for Ger (AN_Ca_2117), Ituchi (AN_Ca_2114), Pascià (AN_Ca_2110) and AN_Ca_0097 and absence in the remaining two genotypes.

4.3 CONCLUSION AND PROSPECTIVES

Given many protocols adopted to extract proteins from plant seeds, which are notoriously with lower protein content than animal tissues and rich in recalcitrant compounds that limit extraction yield, an efficient protocol to extract proteins from chickpea plant tissues has been

described in this work. TCA extraction method followed by solubilization in SDS is more efficient than phenol extraction, regarding chickpea seeds. This method is faster and safer, and a greater number of bands can be noticed by SDS-PAGE.

Very interestingly, by applying TCA protein extraction method followed by SDS-PAGE analysis in six different chickpea genotypes it was possible to identify differences in protein content and protein profiles. This result indicates that it will be really interesting to apply such methodology to phenotype for quality traits (protein content and protein profiles) wide collection of genotypes grown in appropriate replicated experiments to highlight differences among genotypes and how much of this variation is due to genotype and not to the environment. This will allow to both identify interesting genotypes for breeding aimed to developed high quality varieties and to combine such phenotypic evaluation with genetic data to understand the genetic control of protein content and profiles.

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