ABSTRACT

Legumes constitute a significant protein source in the human diet. Among this category, the chickpea (*Cicer arietinum* L.) is recognized as an alternative crop due to its efficient water usage. This efficiency has generated a growing interest in its improvement, commercialization and generation of value-added products, particularly within the domain of functional foods. The literature contains a multitude of techniques for evaluating protein content in legumes, complicating the comparison of results among different research groups. The aim of this study was to assess the effect of four distinct extraction buffers on protein concentration in extracts of chickpea flour from two varieties, Costa 2004 and Blanoro, testing the effect of the extraction temperature at 25 and 37 °C. Additionally, the integrity of the samples was evaluated by analyzing the protein profile using SDS-PAGE. The results obtained showed a significant difference in protein concentration due to the choice of extraction buffer and extraction temperature. We propose the implementation of Tris buffer 500 mM pH 6.8 at 25°C to standardize the process of characterizing protein extracts from chickpea flour for comparative purposes. This standardized protocol
serves as a valuable tool for agronomic characterization and offers a comprehensive framework for future research in this field.

Keywords: extraction buffer, Cicer arietinum L., protein quantification, SDS-PAGE.

1 INTRODUCTION

The chickpea (Cicer arietinum L.) is the third most widely cultivated dry grain legume globally, after beans and peas, in terms of production. In 2016, worldwide chickpea production reached 12 million metric tons, with India, Australia, Myanmar, Pakistan and Turkey being the leading producers; Mexico ranked ninth with 121,567 metric tons (FAOSTAT, 2018).

Chickpea are extensively consumed across the world, and is considered a good source of carbohydrates, proteins, dietary fiber, vitamins and minerals that are readily available and low cost. Moreover, chickpeas are among the legumes with the lowest content of antinutritional factors (Jukanti et al., 2012). When combined with cereals in appropriate proportions, it results in a protein with an improve amino acid profile (Cota et al., 2010).

From an agronomic perspective, chickpeas offer an interesting alternative to traditional crops. They enhance soil fertility by efficiently fixings atmospheric nitrogen through a symbiotic association with rhizobacteria (Wakeyo, 2012). Furthermore, chickpea cultivation requires low water usage, when compared to other crops within the same agricultural cycle, such as wheat and barley (Bolaños-Gonzalez, M., Palacios-Velez, E. & Exebio-Garcia, 2001; Viera de Figueiredo et al., 2013).

Given its importance as a protein source for developing nations, it becomes imperative to sustain high chickpea crop yields. Consequently, genetic improve are essential to achieve these objectives.

In Mexico, the National Institute of Forestry, Agriculture and Livestock Research (INIFAP), consistently focuses on the genetic improvement agronomically valuable species, including chickpea. INIFAP has developed some of the most widely utilized chickpea varieties in the country, such as Blanco-Sinaloa 92, Costa 2004, Jumbo and Blanco Noroeste, while also conducting ongoing assessments of various experimental lines (Ortega-Murrieta et al., 2016).

The genetic improvement of the chickpea is based mainly on agronomic criteria. Given that chickpeas are legumes commonly paired with cereals to achieve a more balanced profile of essential amino acids, it becomes crucial to gather nutritional data (Cota et al., 2010). To facilitate this selection process, it is necessary to have simple, rapid and standardized test that enable the initial evaluation of seed protein content in newly generated lines from breeding programs.

Traditionally, protein content quantification involves methods like Kjeldahl technique, or initial protein isolation followed by quantitative spectrophotometric approaches. Both strategies require
significant sample processing, consuming substantial financial and time resources (Chang et al., 2012; Qayyum, Butt, & Anjum, 2012). While some authors have proposed simplified extraction techniques (Kakaei et al., 2012; Ranjan et al., 2012), these methods have not been widely adopted by the scientific community.

The objective of this study was to assess the impact of four different extraction buffers on the protein yield in flour extracts derived from two varieties of chickpea, Costa 2004 and Blanoro. Furthermore, we examined the influence of extraction temperatures set at 25 and 37°C. In addition, the integrity of the samples was evaluated through protein profile analysis using SDS-PAGE.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

The tests were conducted using white chickpea grain from Blanoro (Hoga-12) and Costa 2004 varieties, which were harvested in the 2011-2012 agricultural cycle in the agricultural region known as “Bajío” (Pénjamo, Guanajuato, México).

2.2 PREPARATION OF THE SAMPLE

The grain of both varieties was ground in a manual mill. The flour was sieved through a 1 mm mesh, to obtain fine flour and to eliminate seed coat.

2.3 DETERMINATION OF MOISTURE

The moisture content of each variety’s flour was measured in triplicate, using a moisture analyzer (OHAUS, USA).

2.4 SELECTION OF BUFFERS

To address the varying solubilities of chickpea grain proteins, which include aqueous-soluble albumins, saline-soluble globulins, and dilute alkali-soluble glutelins (Day, 2013), four buffers were chosen: 1) Distilled water (DW), 2) Sodium hydroxide (NaOH) 50 mM, 3) Tris 500 mM pH 6.8, and 4) sodium sulphite 150 mM (SS, Na₂SO₃).

2.5 PROTEIN EXTRACTION

For protein extraction of both chickpea varieties, the following procedure was carried out: 0.05 g of flour was suspended in 0.8 ml of the respective buffer, and the mixture was stirred for 20 minutes at the specified test temperature (25 or 37 °C). A water bath was used to maintain the temperature. Subsequently, the sample was centrifuged at 6000 rpm for 30 minutes in a minicentrifuge (BioRad, USA),
and the supernatant was recovered. The protein extraction was repeated by adding equal volume of buffer to previously extracted flour, with the resulting supernatant collected in the same tube as the previous one. The protein extract was stored at -70 °C until further analysis.

2.6 DETERMINATION OF TOTAL PROTEIN FLOUR

The nitrogen content of chickpea flour was determined by the Kjeldahl method, employing digestion and distillation equipment for Microkjeldahl ( Büchi, Switzerland). Duplicate determinations with two repetitions were carried out for each variety. To obtain the protein value, a conversion factor of 6.25 was applied.

2.7 QUANTIFICATION OF PROTEIN IN THE EXTRACT

The protein concentration in the extract was determined using the Coomassie blue dye method (Bradford, 1976). A calibration curve was elaborated using bovine serum albumin (BSA, BioRad) as the standard. The absorbance of the samples was recorded at 595 nm in a Thermo Scientific Genesys 10S spectrophotometer; this measurement was conducted in triplicate.

2.8 PROTEIN ELECTROPHORESIS IN DENATURING ACRYLAMIDE GELS (SDS-PAGE)

The Lammli technique was used (Sambrook, Fritsch & Maniatis, 1989). An extract volume equivalent to 40 mg of protein was loaded into each well. The gel analysis was performed using the Quantity One software (Biorad, USA).

2.9 STATISTICAL ANALYSIS

The statistical analysis of the data was performed using SAS software version 8.0.

3 RESULTS AND DISCUSSION

Chickpea grain protein primarily comprises globulins (53-60%) and glutelins (19-25%), with a smaller proportion of albumins (8-12%) and prolamins (3-7%) (Day, 2013). In previous studies, it was observed that the chickpea varieties, Blanoro and Costa 2004, have differences in their soluble protein content (Herrera-Flores et al., 2014), with the Costa 2004 variety having a higher concentration.

In an effort to select an appropriate buffer solution that can effectively identify significant differences in protein quantification in chickpea flour extracts, the extraction buffers were chosen to enable the extraction of proteins solubilized by saline (globulins), alkaline (glutelins) and aqueous (albumins) solutions.
Each extraction was conducted at room temperature (25 °C) and at 37 °C, in order to evaluate the effect of temperature on protein concentration in the extract and its interaction with the extraction buffer. Prior to extraction, the crude protein content was determined by Kjeldahl method (with two replications), resulting in an 18.5% content for the Blanoro variety, and 18.8 % for Costa 2004, with no significant difference observed.

The protein concentration in the extracts, determined by the Bradford method, exhibited mean values of 18.06 mg of protein/mL for the Blanoro variety and 17.06 mg of protein/mL for the Costa 2004 variety. The results obtained indicate that buffer 2 (50 mM NaOH) facilitated the highest protein extraction (Table 1).

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Protein concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH 50 mM</td>
<td>21.64 a</td>
</tr>
<tr>
<td>Tris 500 mM</td>
<td>18.74 b</td>
</tr>
<tr>
<td>Distilled water</td>
<td>16.81 b</td>
</tr>
<tr>
<td>Sodium sulphite 150 mM</td>
<td>13.04 c</td>
</tr>
</tbody>
</table>

Values with different letter indicate statistical significance, α= 0.01, LSD: 2.73. Source: Self-elaboration.

Contrary to was expected based on the solubility of the globulins, the Na$_2$SO$_3$ (150 mM) buffer extraction yielded the lowest protein concentration. This outcome may be attributed to the alkaline characteristics of the NaOH buffer, coupled with the presence of sodium, which facilitated the solubilization of both globulins and glutelins (Day, 2013). It has been previously reported that proteins in white chickpea flour exhibit high solubility at alkaline pH (7.0-12.0) (Ghribi et al., 2015).

Regarding the extraction temperature, as expected, there was a higher protein extraction achieved at 37°C compared to the ambient temperature of 25°C. This aligns with the well-established principle that there is a positive correlation between solubility and temperature for most substances (Table 2).

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Protein concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>19.65 a</td>
</tr>
<tr>
<td>25</td>
<td>15.47 b</td>
</tr>
</tbody>
</table>

Values with different letter indicate statistical significance, α= 0.01, LSD: 1.93. Source: Self-elaboration.

In terms of interactions between the extraction solution and temperature, it was observed that distilled water exhibited the highest protein extraction at 37 °C, but when the temperature was 25°, it decreased by almost three times, resulting in the lowest value among the tested buffers (Table 3).
Table 3. Effect of the buffer and temperature interaction on the protein concentration in extract

<table>
<thead>
<tr>
<th>Buffer/Temperature(°C)</th>
<th>Protein concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW/37</td>
<td>24.7 a</td>
</tr>
<tr>
<td>NaOH/25</td>
<td>22.6 ab</td>
</tr>
<tr>
<td>NaOH/37</td>
<td>20.67 bc</td>
</tr>
<tr>
<td>TRIS/25</td>
<td>20.04 bc</td>
</tr>
<tr>
<td>TRIS/37</td>
<td>17.44 dc</td>
</tr>
<tr>
<td>SS/37</td>
<td>15.78 d</td>
</tr>
<tr>
<td>SS/25</td>
<td>10.31 e</td>
</tr>
<tr>
<td>DW/25</td>
<td>8.92 e</td>
</tr>
</tbody>
</table>

DW: Distilled water, NaOH: Sodium hydroxide, SS: Sodium sulphite.
Values with different letter indicate statistical significance, α= 0.01, LDS: 3.87
Source: Own elaboration.

All other buffer solutions exhibit a similar pattern, with higher efficiency for NaOH 50 mM, followed by Tris 500 mM, and the lowest values observed for Na₂SO₃ 150 mM.

When comparing protein concentrations among chickpea varieties under each condition (buffer and temperature), NaOH at 37 °C and Tris at 25 °C showed the largest arithmetic difference between the two varieties (Table 4), with a higher value for the Blanoro variety (22.24 mg/mL) compared to Costa 2004 (18.93 mg/mL). Neither using distilled water (25 and 37 °C), Na₂SO₃ (25 and 37 °C) nor Tris (37 °C), revealed any significant differences in protein concentration between the two varieties (Data not shown).

Table 4. Protein concentration in protein extracts of chickpea varieties

<table>
<thead>
<tr>
<th>Buffer/Temperature (°C)/Var</th>
<th>Protein (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH/37/Blanoro</td>
<td>22.42 ab</td>
</tr>
<tr>
<td>NaOH/37/Costa 2004</td>
<td>18.93 bcd</td>
</tr>
<tr>
<td>TRIS/25/Blanoro</td>
<td>21.19 abcd</td>
</tr>
<tr>
<td>TRIS/25/Costa 2004</td>
<td>18.9 bcd</td>
</tr>
</tbody>
</table>

Values with different letter indicate statistical significance, α= 0.01, LDS: 5.47
Source: Own elaboration.

To assess the stability of the protein extract and the condition of the extracted proteins, an analysis of the protein profile was conducted through electrophoresis in polyacrylamide gels (SDS-PAGE).

The protein solutions extracted with distilled water and sodium sulfite exhibit highly similar profiles (Figure 1), with observable bands that are presumed to correspond to the sizes of the protein subunits found in chickpea grains. The profile show coincidences with proteins of garbanzo grain: Globulins (Legumin: 25 and 40–45 kDa; Vicilin: 15–70 kDa), Glutelines (17, 38 and 51 kDa), Albumins (8 kDa) and Prolamines (Lipoxygenase 91-93 kDa), in accordance with previous reports (Chang et al., 2012).
The number of bands observed was quite consistent across varieties and extraction temperatures, ranging from 14 to 16 bands per lane. However, an exception was noted in the Costa 2004 variety with Na$_2$SO$_3$ at 37 °C, where 19 bands were identified. Conversely, the protein profile for NaOH displayed significant protein degradation (Figure 2), further supported by average of 9-10 bands per lane and 14 for Blanoro at 25°C.

In contrast, the extract with Tris revealed 19 bands (25 °C) and 16 bands (37 °C) for the Blanoro variety, while 20 bands per lane were observed for the Costa 2004 variety. Notably, this extraction solution was the only to exhibit a difference in the protein profile among varieties.
Figure 2. Protein profile (10% SDS-PAGE) of the *C. arietinum* flour extract with NaOH and Tris.


Source: own elaboration, gel analysis was performed using the Quantity One software (Biorad, USA).

4 CONCLUSIONS

The extracts obtained with buffer NaOH 50 mM consistently exhibited a higher protein content, independently of the extraction temperature. However, protein profile analysis indicated some degree of sample denaturation. Overall, a notable interaction between the extraction buffer and the temperature was evident. Specifically, employing distilled water at 37 °C yielded the highest protein concentration, whereas using the same agent at 25 °C resulted in the lowest concentration in protein extracts.

Although there was no statistically significant difference in protein concentration between the Blanoro and Costa 2004 varieties as determined by the Kjeldahl method, the alkaline buffers NaOH 50 mM and Tris 0.5 mM proved to be more effective in solubilizing chickpea grain proteins. Notably, the extraction buffer Tris 0.5 mM emerged as the most viable option for future studies. Even at room temperature, it yielded protein values similar to those obtained with NaOH and higher than distilled water and Na$_2$SO$_3$.

Furthermore, electrophoretic analysis showed that the Tris 500 mM pH 6.8 buffer provided the best definition and highest number of detected bands. This allowed for the establishment of differences in the electrophoretic profile among the evaluated chickpea varieties.
REFERENCES


