Article

Preliminary Study of Extended Ripening Effects on Peptides Evolution and DPPH Radical Scavenging Activity in Mexican Goat Cheese

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Abstract: Cheese ripening causes intense proteolysis, particularly when the cheese contains starter cultures. Several studies have shown the presence of bioactive peptides in goat’s milk cheeses with antioxidant activity. Mexican goat cheeses’ peptide fractions were evaluated at different ripening stages. Additionally, they were correlated with their antioxidant activity. Proteolysis was measured in the acid-soluble nitrogen and non-protein nitrogen fractions using reverse-phase high-performance liquid chromatography. While the antioxidant activity in both nitrogenous fractions was determined using a 2,2-diphenyl-1-picrylhydrazyl solution. Analyzed cheeses showed peptides fraction in the retention time of 2.05, 18.36, and 50.11 min for acid-soluble fraction and non-protein protein nitrogen, and showed antioxidant activity from the first day of ripening to 73% discoloration in the DPPH solution at 55 ripening days. Obtained results suggested that ripened Mexican goat cheese had a DPPH radical scavenging activity related to peptides present originally in the milk or released by starter culture action during cheese ripening.

Keywords: goat cheese; bioactive peptides; DPPH radical scavenging activity; principal component analysis

1. Introduction

Certain foods can produce beneficial effects on the health of those who consume them. These effects are known as bioactivities and are present in vegetal or animal sources. Several authors have reported the presence of protein fractions with bioactivities such as antioxidant activity in the milk case. This effect is related to eliminating reactive oxygen species (ROS) and free radicals produced during oxidative metabolism. These can cause the appearance of degenerative diseases such as Parkinson’s, Alzheimer’s, and cancer [1,2]. Several authors have demonstrated the presence of bioactive peptides derived from hydrolyzed casein’s milk that have antioxidant effects, such as cow’s milk [3,4], buffalo milk [5,6], sheep’s milk [7], and goat’s milk [8,9]. Consequently, dairy products obtain the same effects. Different types of cheeses have been shown to have antioxidant activities regardless of their production. Such is the case of white cheese [10–12], Parmigiano Reggiano cheese [13], and Cheddar cheese [14,15]. Cheese is one of the most consumed dairy products due to its taste and nutritional qualities. Milk proteins undergo destruction processes during cheese production and ripening, either due to the presence of milk enzymes or microorganisms’ effects. This process is known as proteolysis, and it occurs from the moment in which the enzymatic precipitation takes place until the end of the ripening [16,17]. Mexico has an important production of ripening goat cheeses in tropical areas such as Tabasco, Oaxaca, and Veracruz but the little knowledge and distribution contribute to a poor interest, also there are a few studies about their biochemical ripening...
conditions [18]. Growth and feeding conditions in a different geographical area and environmental characteristics as climate, season, and elevation over the sea contribute to altering the protein composition of milk and consequently, the ripening process and bioactive peptides release during cheese making [19,20]. The importance of studying the bioactivities present in Mexican goat’s milk and cheeses contributes to supporting producers and improving the quality of the products since they could be compared with other cheeses produced and studied in different geographical areas. It is considered that the study of the presence of bioactivities in goat’s milk cheeses made in Mexico will give the product greater value by being able to compare it with other international products. This work aims to determine the protein profile and its relation to DPPH radical scavenging activity in cheeses with extended ripening of goat’s milk made in Veracruz.

2. Results

2.1. Analysis of Peptide Evolution

Nitrogen fractions (NPN and ASN) were used to measure the proteolysis during prolonged ripening in Mexican goat cheese. Data were normally distributed. The peaks used for the analysis were selected, considering those presenting an area bigger than 200 A.U. and the presence in at least 30 of the 90 ripening days. Figure 1 shows the chromatograms in two of the ripening stages and the peaks observed in the NPN fraction.

The mean values of % area obtained by ANOVA test (95% of significance) of the frequent peaks in the chromatograms throughout the 90 days of ripening were obtained. For the statistical analysis, peaks appearing on all ripening stages were selected. In Figure 2 could be observed the evolution of the mean peaks of ASN and in Figure 3 for NPN. For ASN were retained the peaks at 1.18 ± 0.02, 1.91 ± 0.03, 2.43 ± 0.03, and 106.41 ± 0.07 min. For NPN, the retained peaks were those at 1.12 ± 0.02, 2.15 ± 0.02, 5.19 ± 0.1, and 116.5 ± 0.99 min.

![Figure 1. Cont.](image-url)
Figure 1. Chromatograms corresponding to the NPN fraction. (a) Chromatogram of the first ripening day. (b) Chromatogram of the last ripening day.

Figure 2. The evolution of peptides throughout the ripening process. ASN fraction where 4 main peaks are observed. RT: retention time of the selected peak. * Values in the secondary axis.
Table 1. ANOVA and Fisher’s LSD test (p < 0.05) of % area of hydrophobic (HO), hydrophilic (HI) peptides, and the ratio (HO/HI) in the nitrogenous fractions throughout all ripening processes in Mexican goat cheese.

| Peptide Fraction | Peptide Proportion | p-Value | Day 1   | Day 5   | Day 8   | Day 12   | Day 19   | Day 29   | Day 40 | Day 55 | Day 90 |
|------------------|--------------------|---------|---------|---------|---------|---------|---------|---------|--------|--------|--------|
| NPN              |                   |         | 32.10±0.13 b | 31.42±0.77 ab | 20.60±0.60 bc | 20.60±0.60 bc | 20.60±0.60 bc | 20.60±0.60 bc | 20.60±0.60 bc | 20.60±0.60 bc | 20.60±0.60 bc |
|                  |                   |         | 382.03±0.13 a | 382.03±0.13 a | 382.03±0.13 a | 382.03±0.13 a | 382.03±0.13 a | 382.03±0.13 a | 382.03±0.13 a | 382.03±0.13 a | 382.03±0.13 a |
| H30H23           |                   |         | 299.51±0.13 bc | 309.12±0.13 bc | 309.12±0.13 bc | 309.12±0.13 bc | 309.12±0.13 bc | 309.12±0.13 bc | 309.12±0.13 bc | 309.12±0.13 bc | 309.12±0.13 bc |
| H31257           |                   |         | 499.54±0.13 c | 499.54±0.13 c | 499.54±0.13 c | 499.54±0.13 c | 499.54±0.13 c | 499.54±0.13 c | 499.54±0.13 c | 499.54±0.13 c | 499.54±0.13 c |
|                  |                   |         | 277.30±0.13 d | 277.30±0.13 d | 277.30±0.13 d | 277.30±0.13 d | 277.30±0.13 d | 277.30±0.13 d | 277.30±0.13 d | 277.30±0.13 d | 277.30±0.13 d |
|                  |                   |         | 566.26±0.13 e | 566.26±0.13 e | 566.26±0.13 e | 566.26±0.13 e | 566.26±0.13 e | 566.26±0.13 e | 566.26±0.13 e | 566.26±0.13 e | 566.26±0.13 e |
|                  |                   |         | 970.10±0.13 f | 970.10±0.13 f | 970.10±0.13 f | 970.10±0.13 f | 970.10±0.13 f | 970.10±0.13 f | 970.10±0.13 f | 970.10±0.13 f | 970.10±0.13 f |
|                  |                   |         | 1511.05±0.13 g | 1511.05±0.13 g | 1511.05±0.13 g | 1511.05±0.13 g | 1511.05±0.13 g | 1511.05±0.13 g | 1511.05±0.13 g | 1511.05±0.13 g | 1511.05±0.13 g |
|                  |                   |         | 2884.98±0.13 b | 2884.98±0.13 b | 2884.98±0.13 b | 2884.98±0.13 b | 2884.98±0.13 b | 2884.98±0.13 b | 2884.98±0.13 b | 2884.98±0.13 b | 2884.98±0.13 b |
|                  |                   |         | 8079.73±0.13 c | 8079.73±0.13 c | 8079.73±0.13 c | 8079.73±0.13 c | 8079.73±0.13 c | 8079.73±0.13 c | 8079.73±0.13 c | 8079.73±0.13 c | 8079.73±0.13 c |
|                  |                   |         | 10539.89±0.13 a | 10539.89±0.13 a | 10539.89±0.13 a | 10539.89±0.13 a | 10539.89±0.13 a | 10539.89±0.13 a | 10539.89±0.13 a | 10539.89±0.13 a | 10539.89±0.13 a |
|                  |                   |         | 10110.30±0.13 a | 10110.30±0.13 a | 10110.30±0.13 a | 10110.30±0.13 a | 10110.30±0.13 a | 10110.30±0.13 a | 10110.30±0.13 a | 10110.30±0.13 a | 10110.30±0.13 a |
|                  |                   |         | 9419.64±0.13 a | 9419.64±0.13 a | 9419.64±0.13 a | 9419.64±0.13 a | 9419.64±0.13 a | 9419.64±0.13 a | 9419.64±0.13 a | 9419.64±0.13 a | 9419.64±0.13 a |
|                  |                   |         | 10160.42±0.13 a | 10160.42±0.13 a | 10160.42±0.13 a | 10160.42±0.13 a | 10160.42±0.13 a | 10160.42±0.13 a | 10160.42±0.13 a | 10160.42±0.13 a | 10160.42±0.13 a |
|                  |                   |         | 10120.08±0.13 a | 10120.08±0.13 a | 10120.08±0.13 a | 10120.08±0.13 a | 10120.08±0.13 a | 10120.08±0.13 a | 10120.08±0.13 a | 10120.08±0.13 a | 10120.08±0.13 a |
|                  |                   |         | 16480.31±0.13 c | 16480.31±0.13 c | 16480.31±0.13 c | 16480.31±0.13 c | 16480.31±0.13 c | 16480.31±0.13 c | 16480.31±0.13 c | 16480.31±0.13 c | 16480.31±0.13 c |

* Different letters in values of the same fraction are significantly different (p < 0.05). Each letter represents a different group obtained in Fisher’s LSD test.

2.3. DPPH Discoloration Percentage Evolution

The % of DPPH discoloration shows the antioxidant effect in the evaluated fractions. The increase in the discoloration demonstrates higher antioxidant activity in the tested substance. Figure 4 shows the evolution of antioxidant activity during all ripening processes in both fractions.

2.4. PCA Analysis NPN Fraction

Figure 5 shows the PCA analysis carried out on the NPN fraction. Figure 5a shows the factorial map of the peptides HO, HI, the relationship between these, and DPPH. They were defined as PC1 and PC2, and the behavior was explained in 59.28% and 36.50%, respectively. Samples were separated into three groups. The first one grouped cheeses from day 1 to day 12, corresponding to young cheeses. The second group grouped cheese from day 19 to day 55, corresponding with the commercial ripening time. At the same time, the third group corresponds to the extended ripened cheeses at 90 days. Figure 5b shows
the loadings plot HI/HO showed a strong correlation with PC1, while individually, HO and HI were correlated with both axes, PC1 and PC2.

Figure 4. Evolution of DPPH discoloration percentage during the ripening process in both fractions.

Figure 5. HI, HO, ratio HO/HI, and antioxidant activity in NPN profile. Two principal components (PC1 and PC2) are observed in PCA plots. (a) Factorial map obtained by principal components analyses (PCA) all in samples obtained by controlled ripening system. Arrows show the trend followed throughout the ripening process. (b) Factor loading plots (PC1 and PC2). Samples code represents the ripening day.

2.5. PCA Analysis ASN Fraction

Figure 6 shows the PCA analysis carried out on the ASN fraction. Figure 6a shows the factorial map of the peptides HO, HI, the relationship between these, and DPPH. They were defined by PC1 and PC2 that explained 51.61% and 43.15% of the total variance, respectively. In Figure 5a could be observed three main groups, one showing the first 29 days of ripening corresponding with young cheeses. Another is observed for the 40th
ripeing day corresponding to the commercial ripening time, and the last group, grouping cheeses from the 50th to 90th ripening day corresponding to the most ripened cheeses. Figure 6b shows the loadings plot. HO/HI ratio was strongly and negatively correlated with PC1. At the same time, HI and HO were correlated with both axes.

Figure 6. HI, HO, ratio HO/HI, and antioxidant activity in ASN profile. Two principal components (PC1 and PC2) are observed in PCA plots. (a) Factorial map obtained by principal components analyses (PCA) all in samples obtained by controlled ripening system. Arrows show the trend followed throughout the ripening process. (b) Factor loading plots (PC1 and PC2). Samples code represents the ripening day.

2.6. Correlation Analysis

Table 2 shows the correlation between the individual peptides found in the ASN fraction, the HO and HI peptides, the HO/HI ratio, and the antioxidant effect found in this fraction. A highly significant correlation ($p < 0.05$) can be observed between antioxidant activity and the peak at 2.43 min. Table 3 shows the correlation between the peptides found in the NPN fraction, the HI and HO peptides, the HO/HI ratio, and the antioxidant effect found in this cheese fraction. Highly significant correlations were found between the relationships between retention times and the HO/HI ratio.

Table 2. Correlation values for retention time, peptides proportion, and antioxidant effect in ASN fraction in Mexican goat cheese with prolonged ripening.

| Variables | RT 1.18 | RT 1.91 | RT 2.43 | RT 106.41 | HI     | HO     | HO/HI |
|-----------|---------|---------|---------|-----------|--------|--------|-------|
| RT 1.91   | 0.27 ** |         |         |           |        |        |       |
| RT 2.43   | 0.24 *  | 0.46 ***|         |           |        |        |       |
| RT 106.41 | 0.07    | 0.36 ***| 0.08    |           |        |        |       |
| HI        | 0.32 ** | 0.93 ***| 0.75 ***| 0.30 **   |        |        |       |
| HO        | 0.07    | 0.36 ***| 0.08    | 0.99 ***  | 0.30 **|        |       |
| HO/HI     | −0.19   | −0.54 ***| −0.28 **| 0.44 ***  | −0.51 ***| 0.44 ***|       |
| DPPH      | −0.15   | 0.16    | 0.31 ** | −0.05     | 0.25 * | −0.05 | −0.16 |

Notes: * Correlation values significant at $p < 0.1$; ** significant at $p < 0.05$; *** significant at $p < 0.01$. 


Table 3. Correlation values for retention time, peptides proportion, and antioxidant effect in the NNP fraction in Mexican goat surface mold cheese with prolonged ripening.

| Variables | RT 1.12 | RT 2.15 | RT 5.19 | RT 116.57 | HI    | HO     | HO/HI   |
|-----------|---------|---------|---------|-----------|-------|--------|---------|
| RT 2.15   |         | 0.03    |         |           |       |        |         |
| RT 5.19   | 0.30 ** |         | −0.08   |           |       |        |         |
| RT 116.57 | 0.23 *  | 0.11    | −0.19   |           |       |        |         |
| HI        | 0.07    | 0.99 ***| 0.01    | 0.10      |       |        |         |
| HO        | 0.23 *  | 0.11    | −0.19   | 0.99 ***  | 0.10  |        |         |
| HO/HI     | 0.19    | −0.64 ***| −0.10   | 0.51 ***  | −0.65 ***| 0.51 ***|         |
| DPPH      | 0.11    | 0.13    | −0.10*  | 0.15      | 0.12  | 0.15   | 0.08    |

Notes: a Correlation values * significant at \( p < 0.1 \); ** significant at \( p < 0.05 \); *** significant at \( p < 0.01 \).

3. Discussion

The behavior of the two protein fractions’ values shows the release of peptides throughout the ripening process. ASN and NPN fractions showed a significant increase in the peptides fraction mentioned above from day 1 to day 90, which can be observed in Figures 2 and 3. There is a fluctuating behavior in both fractions in the area of the peaks between days 8 and 40 of ripening. This can be explained by the presence of proteolytic enzymes found in milk as plasmin and rennet, which promote the release of medium and short-chain peptides from \( \beta \)-caseins. Subsequently, a tendency to a significant increase in the peaks’ areas is observed between days 40 and 90 of ripening. This is related to the release of the same peptides thanks to peptidases produced by the microorganisms developed throughout the ripening process.

These results are related to the reported by Fox, P. et al. [21], Ardö, Y. et al. [22] and McSweeney, P. L. [23], who observed that intense proteolytic activity is not only due to the microorganisms during the ripening process, caseins are hydrolyzed thanks to the effect of digestive proteases contained in the food matrix. The proteins produced by these mechanisms are hydrolyzed to short peptides and free amino acids [22,24]. This effect is observed in long ripened cheeses as in the work elaborated by Barać, M. et al. [11] in white cow cheese where an increase in water-soluble fraction to 1.88 to 14.46 g/100 mg was observed; Gupta, A. et al. [14] in Cheddar cheeses at different stages of ripening; Huma, N. et al. [15] in Cheddar cheeses 6 months ripened, and Basiricó Basiricò, L. et al. [25] in Parmigiano Reggiano cheese with 12 ripening months.

The proportion of hydrophobic peptides with hydrophilic peptides (HO/HI) indicates a greater quantity of peptides released during all ripening stages and that there were increases in them near the 90th ripening day and is an important parameter that can indicate the proteolysis characteristics, particularly in the kind of peptide released and is significantly related to ripening process [26]. The formation of the HI fraction indicates the presence of medium-sized peptides that are usually soluble in water and frequently produced by the presence of peptidases produced by starter and non-starter microorganisms, unlike the HO fraction, which is represented by long peptides produced by the enzymes obtained by the rennet and enzymes found in the cheese matrix [26,27]. The obtained results show an increase in the parameters of hydrophilic peptides and hydrophobic peptides in both fractions. Fisher’s posthoc analysis shows no differences between groups in almost all ripening processes; however, it can be observed that there is a much higher amount of hydrophilic peptides related to the presence of microorganisms that promote the proteolysis during the 90 ripening days. On the other hand, proteolysis’s presence due to enzymes in the product matrix is observed to a lesser extent.

These results can also be observed in Figures 2 and 3, where hydrophilic peptides (less than 35 min of retention time) are found in a greater proportion compared to hydrophobic peptides (more than 35 min of retention time). Correlation analysis observed in Tables 2 and 3 shows a highly significant correlation between HI, HO, and retention times in both fractions. The results show the presence of proteolysis that is directly related...
to the ripening process. Tejada, L. et al. [28] also reported this evolution. They found the presence of hydrophilic peptides in Murcia al Vino goat cheese with up to 60 days of ripening, observing a decrease in ratio HO/HI during all ripening processes, with fluctuations in values from 8.5 from the first ripening day to 5.5 to 60th ripening day without significant differences. On the other hand, there are the studies elaborated by Hernández-Galán, L. et al. [16] and Vivar Quintana, A. M. et al. [26], who observed a high value in the HO/HI ratio due to the presence of hydrophobic peptides after analyzing unripened goat, cow, and ewe cheeses.

The presence of antioxidant activity was observed from the first day of analysis. Figure 4 shows the evolution during all ripening processes in both fractions. Although both fractions showed antioxidant activity, the NPN fraction showed higher activity throughout ripening, significantly increasing after day 55, 0.02 \( p \)-value in ANOVA test and grouping differences in Fisher LSD test (0.95 confidence interval). The NSA fraction showed the same antioxidant activity during the entire ripening without significant changes. Correlation analysis observed in Table 2, where the ASN fraction is analyzed, shows a highly significant positive relationship between DPPH activity and retention time 2.43. The NNP fraction correlation analysis showed a significant positive relationship between DPPH activity and retention time 5.19, which indicates that the compounds that promote the activity are in compounds observed in the hydrophilic fraction.

Several authors have observed the presence of antioxidant activity in milk and cheese in water-soluble fractions and medium or short peptides [12,13]. On the other hand, it is known that whole milk, such as cow, goat, and buffalo milk, has antioxidant activity and that after the production process and cheese ripening, no significant variations in antioxidant activities [8,13]. This behavior has been observed in different types of cheeses that have exhibited activity, such as Barač, M. et al. [11], who observed the presence of constant antioxidant activity in white cheese with a percentage of antioxidant effect over 50% in water-soluble protein fraction and over 15% in non-water-soluble protein fraction after 50 ripening days; Bottesini, C. et al. [13] in hydrophilic fractions of Parmigiano Reggiano cheese with a no significative difference in antioxidant effect after 41 months in the ripening process, with a maximum of 13 \( \mu \)mol/mg of protein in TEAC during the first 24 ripening months. For Cheddar cheese, Gupta, A. et al. [14] and Huma, N. et al. [15] observed the antioxidant effect during 9 and 6 months, respectively, without significant variations in hydrophilic fractions. On the other hand, Meira, S. M. M. et al. [29] observed the effect using the DPPH test in a hydrophilic fraction on Roquefort cheese made from sheep’s milk.

The principal component analysis made it possible to explain the 95.78% of total variability with the first two components. In the factor loading plots, an inversely proportional relationship between hydrophilic peptides and hydrophobic peptides indicates that the ripening process in this cheese promotes the main appearance of hydrophilic peptides, which can be compared with what is observed in the ANOVA and correlation tests mentioned above and that agree with what was found in the literature. On the other hand, it is observed that there is a relationship between the antioxidant activity with the principal component 1 in the tested fractions (ASN and NPN), which confirms that the fractions present close relationships with the bioactivity analyzed. This behavior has been observed in other cheeses of prolonged maturation, where retention times corresponding to hydrophilic peptides with the presence of antioxidant activity are observed [11–15,29].

Likewise, the principal component analysis factorial map shows the antioxidant activity’s behavior that presents a trend from the first to the second component during the first days of ripening, followed by a trend toward the second component in the middle and last ripening days.
4. Materials and Methods

4.1. Cheese Production

For this study, 15 primiparous goats’ free-grazing feeding (grass, shrubs, shoots, and leaves of local plants such as *Brugmansia candida* and *Brugmansia suaveolens*) were selected in Pacho Viejo, Coatepec, México (at 1199 m above sea level). Three batches of cheeses were produced in spring: April, May, and June to avoid climate and goat’s alimentation variations. The 15 goats were milked for its batch, milk was mixed, and cheeses were produced the same day in the ranch. The milk was pasteurized at 65 °C for 30 min. The milk was allowed to cool, bovine renin rennet (Coagulmex Co., Veracruz, Mexico) was added in a concentration of 1%. It was left to rest for 8 h to remove the whey. The paste obtained was left scrubbed for 2 more hours, it was kneaded, and the strains of *Staphylococcus lactis*, *Staphylococcus cremoris*, and *Penicillium candidum* were added, taking into account the concentration set by the manufacturer. Once the paste was dry, the fresh cheeses were made in a cylindrical shape (about 5 cm diameter and 15 cm height) with a hand press. Each cheese weight was 150 g. Every batch gets 27 pieces of cheese, a total of 81 pieces of cheese for all experimental work. They were transferred to Tecnologico de Monterrey facilities on the same day of production under refrigeration at 4 °C.

4.2. Ripening Process

To carry out the ripening process, the cheeses were placed in an electronic ripening chamber brand Memmert HPP 260 (GmbH Co, Germany), programming constant temperature between 13 and 14 °C, constant humidity values of 85%, airspeed moderate, and ammonic gas output, conditions that no affect the inoculated microflora growth [18]. Sample withdrawal was carried out on the 1st, 8th, 12th, 19th, 29th, 40th, 55th, and 90th ripening days. On each sampling day, three pieces of cheese were removed and placed at −40 °C until analysis.

4.3. Sample Preparation

Each cheese was grated into small pieces. They were homogenized, and 10 g of the sample were taken and subsequently dispersed in 10 mL of deionized water in a 1:1 ratio. The dispersion was carried out using an Ultra-turrax homogenizer (IKA T18 basic instruments, Germany) for 5 min at 25,000 rpm. The obtained homogenate was incubated in a water bath at 40 °C for one hour, followed by a second homogenization of 5 min at 25,000 rpm. The suspension was centrifuged (Heraus X1, Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 4 °C and 3000 rpm. From the product obtained, the solidified fat was separated by removing it with a spatula. The remaining sample was homogenized for 2 min at 25,000 rpm [30]. The cheese homogenate (CH) was stored at −20 °C until analysis.

4.4. Nitrogen Fractions

To evaluate the peptide fraction of the analyzed cheeses, a physicochemical fractionation was carried out. Before fractionation, the CH was diluted by adding 11.25 mL NaCl (9 g/L) to 1.25 g of CH. This solution was homogenized for 5 min at 25,000 rpm. The diluted cheese homogenate (DCH) was fractionated. Two nitrogen fractions were obtained: non-protein nitrogen (NPN) and acid-soluble nitrogen (ASN) at pH 4.6.

4.4.1. Non-Protein Nitrogen (NPN)

The method described by Leclercq-Perlat, M.-N. et al. [31] was used. A 10 mL aliquot of DCH was added with 10 mL of a trichloroacetic acid solution (240 g/L). Then, it was homogenized at 25,000 rpm for 2 min. This solution was incubated in a water bath for 1 h at 25 °C, then centrifuged at 4000 rpm at 7 °C for 10 min. The supernatant corresponding to the NPN fraction was then filtered using Whatman No. 42 paper and conserved at −18 °C until analysis.
4.4.2. Acid-Soluble Nitrogen (ASN)

To obtain the ASN fraction, the method described by Hernández-Galán, L. et al. [16] was applied. A 10 mL aliquot of CDH was adjusted to pH 4.6 with a 2 N hydrochloric acid solution and incubated in a water bath at 25 °C for 20 min. This solution was centrifuged for 45 min at 6000 rpm at a temperature of 7 °C. The supernatant corresponds to the ASN fraction. It was filtered using Whatman No. 42 paper and conserved at −18 °C until analysis.

4.5. Determination of Peptide Fraction

To determine the peptide fractions, the method described by Hernández-Galán, L. et al. [16] was used. A reverse-phase high-performance liquid chromatography (Agilent Technologies, Model 1200 Palo Alto, CA, USA) with a diode array detector (DAD) was used. The column was a Zorbax Eclipse XDB-C18 (5 µm, 4.6 µm i.d. × 150 mm, Agilent) at an elution rate of 0.75 mL/min in a two-solvent system. (Phase A) 100 mL/L acetonitrile and 0.5 mL/L TFA in HPLC grade water. (Phase B) 600 mL/L acetonitrile and 0.5 mL/L TFA in HPLC grade water.

Samples were initially eluted with 100% solvent A for 10 min. Subsequently, in a linear gradient, B was integrated from 0% to 49% by 98 min and 50% to 80% up to 108 min, followed by a linear gradient from 80% to 100% of B for 5 min. Once 100% was reached, B was maintained for 5 min. The wavelength used in the DAD detector was 215 nm. The injected sample volume was 10 µL, all samples were run in duplicate.

For each chromatogram, the % Area of each peak was obtained, and the compounds were coded with their retention time. The relationship between hydrophilic and hydrophobic peptides was determined following the method defined by De Llano, D. G. et al. [32], where it was established that the first 10 to 35 min of running are observed peptides with hydrophilic characteristics, and from 35 to 120 min, those peptides with hydrophobic tendencies are retained. These data were obtained using the peaks’ areas and following the formula Hydrophobic peptides/Hydrophilic peptides.

4.6. Determination of Antioxidant Activity on 2,2-Diphenyl-1-picrylhydrazyl (DPPH)

For this determination, the method described by Hernández-Galán, L. et al. [33] was applied. An aliquot of 0.02 mL of NPN and ASN fractions were taken and placed into a 96-well flat-bottom plate; they were added with 0.22 mL of DPPH solution (125 µM DPPH in 800 mL/L methanol). The plate was covered with aluminum foil to avoid light decomposition and kept at room temperature for 90 min. Once the time had elapsed, it was read at 520 nm in a UV-Vis spectrophotometer (X Mark Microplate Reader, Bio-Rad Laboratories, Inc., Japan). Results were expressed as the percentage of discoloration, and reagent grade methanol (Karal, Leon, Gto. Mexico) was used as a blank.

4.7. Statistical Analysis

The Statistica v 12 software (Statsoft, Inc., Tulsa, OK, USA) was used to analyze the results. All chromatographic data were separated in the following order: 1. % Area of individual peaks of the NPN fraction; 2. % Area of individual peaks in the ASN fraction; 3. Area ratio between hydrophilic and hydrophobic peptides; 4. Antioxidant activity in the ASN and NPN fractions. Analysis of variance (ANOVA) was applied to all data to evaluate significant statistical differences ($p < 0.05$) between days of ripening. If data were not normally distributed, a Kruska–Wallis test was applied. Subsequently, a Fisher’s least square determinant test (LSD) was carried out to find differences between groups. Correlation analyses were carried out to find correlations between peptides and antioxidant activity. Finally, a principal component analysis (PCA) was carried out to determine the behaviors throughout all ripening times of HI, HO, and the ratio HO/HI. For this analysis, DPPH was considered as supplementary data.
5. Conclusions

The presence of peptides produced in the non-protein and acid-soluble nitrogenous fractions was determined after the prolonged ripening of Mexican goat cheese throughout extended ripening. These peptides showed evidence of producing antioxidant activity, which was observed in both fractions throughout the entire ripening of the cheese. However, it is required to perform a deep analysis to determine which peptide promotes de activity and other antioxidant tests. The study of bioactive peptides in Mexican cheeses gives rise to deep knowledge about the biochemical qualities of ripening in local cheeses. Further studies must be carried out to quantify and sequence the peptides as well as to evaluate the individual antioxidant activity after peptides fractionation.

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