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Chapter 8

INHIBITORY EFFECT OF ANGIOTENSIN-CONVERTING ENZYME AND ANTIOXIDANT CAPACITY OF PANELA TYPE CHEESES

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ABSTRACT

Hypertension is a disease that affects more than 70% of the worldwide population. In Mexico, panela cheese is one of the most consumed cheeses and determinating the antioxidant activity, and angiotensin-converting inhibition effect was the main objective of this study.

We analyzed two different cheese types (A, B) for 15 days and then classified them in 7 samples. The samples were treated and obtained in two fractions, nitrogen soluble in ethanol (ETOH-SN), and nitrogen no-soluble in ethanol (EOTH-NSN). After the treatment, we did the detection of ACEI using HPLC, and the antioxidant activity by DPPH

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method at different concentrations (0.01,0.05,0.12,0.25,0.5 y 1) using a dose-response curve to calculate the IC50.

Our results demonstrate that the ETOH-SN fraction does not present significative differences between batches, but there is a difference compared with ETOH-NSN ($P < 0.05$). Additionally, we determined the antioxidant activity of batch A ETOH-SN, in which sample A3 has a higher inhibition.

We can infer with the antioxidant activity, and the inhibition of ECA that the consumption of panela-type cheeses might be considered a valuable tool against different human diseases, so including this Mexican cheese in the diet could be a good alternative for patients with cardiovascular treatments, such as hypertension.

Keywords: angiotensin-converting, protein hydrolysates, antioxidant activity, panela cheese, hypertension, cheese

INTRODUCTION

Cardiovascular diseases (CVD) contribute mostly to morbidity and mortality worldwide (Alwan et al. 2000), and among these, ischemic heart disease is the leading cause of death according to WHO, (2016) included Hypertension that is a significant risk factor for CVD, and it was estimated that 16.5% of all deaths could be attributed to high blood pressure (BP) (Lim et al. 2012). From prospective observational studies, it was estimated that if there is a reduction of 5 mmHg in diastolic blood pressure, it will reduce the risk of stroke by 34% (MacMahon et al. 1990).

Hypertension is mainly treated with drugs that help stabilize or regulate pressure (Chen, 2018). Within the pharmacological treatment for hypertension are beta-blockers (Wiysonge et al. 2017); the use of ACE inhibitors is preferred due to the reduction of adverse effects in patients (Solomon and Taler 2018). ACE is a dipeptide that activates angiotensin II, which has a vasoconstrictor effect, so there is an increment in blood flow and, as a consequence, an increase in blood pressure (Nilsen et al. 2016; Ali et al. 2017; Stuknyte et al. 2015). On the other hand, there are more alternatives than pharmacological treatment of pressure, such as changes in lifestyle, diet, weight reduction, and low sodium consumption (Solomon and Taler 2018). Those alternatives are useful in the prevention and treatment of hypertension (Appel et al. 2006). This set of changes can be applied to everyday life and used as a preventative strategy for managing not only hypertension but also other diseases related to bad daily habits (Pisano and González 2014).

The detection of healthy habits at an early age could be solved through proper patient education (Sosa-Rosado 2010). Among the most significant changes include maintaining regular weight control, reducing alcohol consumption, eliminating the use of tobacco, changing dietary habits, compliance with nutritional patterns or diets that help lower blood pressure (Soto 2018). The main nutritional plan currently used for patients with hypertension is the DASH diet (Dietary approaches to stop hypertension) (Steinberg,

Bennett, and Svetkey 2017). This diet emphasizes the consumption of fruits, vegetables, healthy fats, and low-fat or non-fat dairy products (Appel et al. 2006; Wiysonge et al. 2017). The increase of fruits, vegetable consumption for their fiber, and the reduction of red meats, starches, added sugars, processed foods are the premises of this diet (Bes-Rastrollo 2017), all this in conjunction with a decrease in sodium intake that will be important for the treatment and prevention of hypertension (Sacks et al. William 2001). There are other diets such as the Mediterranean, which shares a similarity in terms of its composition with the DASH (Sánchez-Tornel 2017) since it also promotes the varied consumption of foods of vegetable origin. Olive oil is used in the Mediterranean diet, as the primary and only lipid source for culinary preparations, the wine consumption is moderate and distributed throughout the week, and dairy products such as yogurt and cheese are consumed (Bes-Rastrollo 2017). The Mediterranean diet has shown a reduction in the risk of CVD, as well as prevention and reduction of blood pressure (Zanuy-Válero 2013). The vegetarian diet also uses olive oil as a lipid content, promotes the varied consumption of vegetables (Morales-Valdez et al. 2015), in addition to adding eggs and dairy to its composition, it has a positive impact on blood pressure due to its contribution of fiber, vegetable protein and nutrients such as magnesium and potassium (Richter et al. 2015).

The consumption of dairy products is a common factor and can be found among the diets recommended for the prevention and treatment of hypertension. Commonly, the global population consumes these products for their content of proteins, minerals (calcium, magnesium, phosphorus, and potassium) and vitamins (D, folates) (McGrane et al. 2011; Sacks et al. 2001)**.** Currently, some studies support non-pharmacological therapeutic interventions (ITNF), based on the restriction of dietary sodium consumption (Zanuy-Válero 2013). Antihypertensive treatment that uses drugs has had low success in reducing vascular complications (Nguyen et al. 2010), on the other hand, non-pharmacological measures are cheap and generate benefits that promote a better state of health (De La Sierra et al. 2008). Also, the excellent contribution of nutrients provides us amino acids that, through digestion produced by the action of a combination of proteases of milk and protease secreted by the intestinal tract release fragments of proteins (peptides) that can present specific biological activities (Beverly, Underwood, and Dallas 2019).

Bioactive peptides (BP) are short-chain peptides derived from the intact protein and have multiple physiological functions (Aslam et al. 2019) presented in cheese fraction. These peptides presented in cheese fractions can be used as functional food ingredients, or nutraceuticals and pharmaceuticals to improve human health. These activities include immunomodulatory, anti-hypersensitive, antimicrobial, osteoprotective, and opioid functions. The effects of these peptides are associated with their specific content and the constitution of amino acids (Zanutto-elgui et al. 2018; Aslam et al. 2019).

For this reason, the benefits of the consumption of dairy products should not only be attributed to the nutrient content but also due to the release and function of the bioactive peptides in the body.

The release of these peptides varies depending on the types of cheeses, their manufacturing process, the external components added, and the stage of digestion (Akbari-adergani 2019). Concerning bioactive peptides that have antihypertensive activity, among its main health benefits is the inhibition of the angiotensin-converting enzyme (ACE) (Han, Maycock, and Murray 2018; Stuknyte et al. 2015).

These peptides mainly acquire their effect by the inhibition of the angiotensin-converting enzyme (ACE) (Stuknyte et al. 2015). The angiotensin-converting enzyme (peptidyl dipeptide hydrolase, EC 3.4.15.1) is an exopeptidase that cleaves the C-terminal dipeptides of several oligopeptides. As part of the renin-angiotensin system. ACE hydrolyzes angiotensin I, an inactive decapeptide, to the potent vasoconstrictor angiotensin II. It also activates the bradykinin vasodilator, which participates in the control of blood pressure (Korhonen and Pihlanto 2006).

Table 1. Composition of several fresh brands of panela cheese (%)

Mexican brands	Moisture (±0.5%)	Fat (±0.5%)	Protein (±0.5%)	Calcium mg/100g
Aguascalientes	52	22	22	818
Alpura	54	22	19	716
Chilchota	51	26	19	688
Ixtacalco	56	22	19	633
Caperucita	54	23	18	644

(National Laboratory for Consumer Protection 2007).

Recent research shows interest in the study of Latin American cheeses due to their latent content of bioactive peptides (Fuentes-García 2017; Martin and Deussen 2017). Cheese is one of the derivatives of milk more frequently consumed worldwide. Cheese is easy to digest and well-tolerated; it is estimated that each person consumes around 15.5 kg of cheese per year (42.5 g/d) in the United States and 19.7 kg in Europe (49.0 g/d) (Canadian Dairy Information Centre 2014). In addition to this, other studies indicate that an approximate consumption between 50-60 g/d can provide a cardioprotective effect (Chong et al. 2016).

Among the cheeses that stand out are the fresh cheeses like the panela-type fresh cheese, also known as “panela style” or “panela basket.” The cheese is obtained from the separation of the casein from whey by the action of the rennet and/or specific enzymes, which gives rise to a soft substance called curd, which, once stable, is pressed and drained to finish separation of the whey.

Panela cheese is one of the most popular and consumed cheeses in Mexico due to its mild slightly salty taste, and because consumers consider it "healthy" due to its low-fat content (National Laboratory for Consumer Protection 2007). Compared to the different Mexican brand of panela cheeses and the nutritional composition, there all have similar nutritional composition regardless of the Mexican brands, Table 1.

The fresh Mexican cheese is characterized by being high in moisture content, having a mild-milky flavor, a soft and creamy texture, and a very short shelf-life (Van Hekken et al. 2010). This type of cheese has a moisture content of 63.4 ± 3.8%, the protein content of 15.63 ± 1.35%, the fat content of 15 ± 3.16%, and a pH of 6.0 ± 0.2 (Torres-Llanez et al. 2011). As we mentioned before, the bioactive peptides presented in cheese fraction has an IECA activity, and this peptide is produced during the manufacturing process and even after their useful life (Tarango-Hernández et al. 2015). Based on all the above, we can infer that, when treating diseases such as hypertension, it is essential to include several foods to our diet, following the different diets mentioned such as DASH, Mediterranean and vegetarian diet (Pérez-fuentes 2015). Besides, we should include in our natural diet dairy milk products like cheese (Rowland et al. 2002), for its nutritional value and the presence of biologically active components as α-lactalbumin, β-lactoglobulin, lactoferrin, lactoperoxidase and serum albumin (Ebringer, Ferencik, and Krajcovic 1986).

Finally, fresh cheese presented also an antioxidant activity, which plays a protective role, reducing the formation of free radicals that help to control and prevent degenerative diseases such as arteriosclerosis, diabetes, hypertension and cancer, whose common etiological factor is oxidative stress (Tarango-Hernández et al. 2015; Torres-Llanez et al. 2011).

Given that, at present, foods play a fundamental role in the prevention and treatment in the field of medicine, cheese is addressed as possible source of dietary antioxidants (Herrera, Betancur, and Segura 2014). This research aims to determine antioxidant activity and antihypertensive activity of bioactive peptides generated in fresh panela cheese.

MATERIALS AND METHODS

Samples

The samples considered in the present study were panela type cheese randomly collected from different markets of Querétaro City, México. We analyzed two types of cheese A: Chilchota (23 samples) and B: Caperucita (28 samples) for 15 days. The cheeses A and B were classified into seven samples. These samples were from a mixture of cheeses from the same type. The number of cheeses used in each sample is shown in Table 2.

Table 2. Cheese number per batch

Type A (23)		Type B (28)	
Code	Cheese (n)	Code	Cheese (n)
1A	8	1B	1
2A	3	2B	3
3A	7	3B	9
4A	2	4B	4
5A	1	5B	1
6A	2	6B	7
7A	1	7B	4

Treatment of Samples/Proteolysis of Samples

The cheeses samples were treated with water and then homogenized, incubated, and centrifugated to obtain the precipitated residue as a Non-Fat Cheese fraction Figure 1, according to the methodology of Guerra-Martínez et al. 2012. This residue was stored at -80°C (Guerra-Martínez, Montejano, and Martín-Del-Campo 2012). The fractionation obtained of the Crude Nitrogen yielded three fractions: Acid Soluble Nitrogen (ASN) at pH 4.6, Non-protein Nitrogen (NPN), and 70% Ethanol-soluble Nitrogen (EtOH-SN), which were evaluated for antioxidant and ACE inhibitory activities (Figure 1).

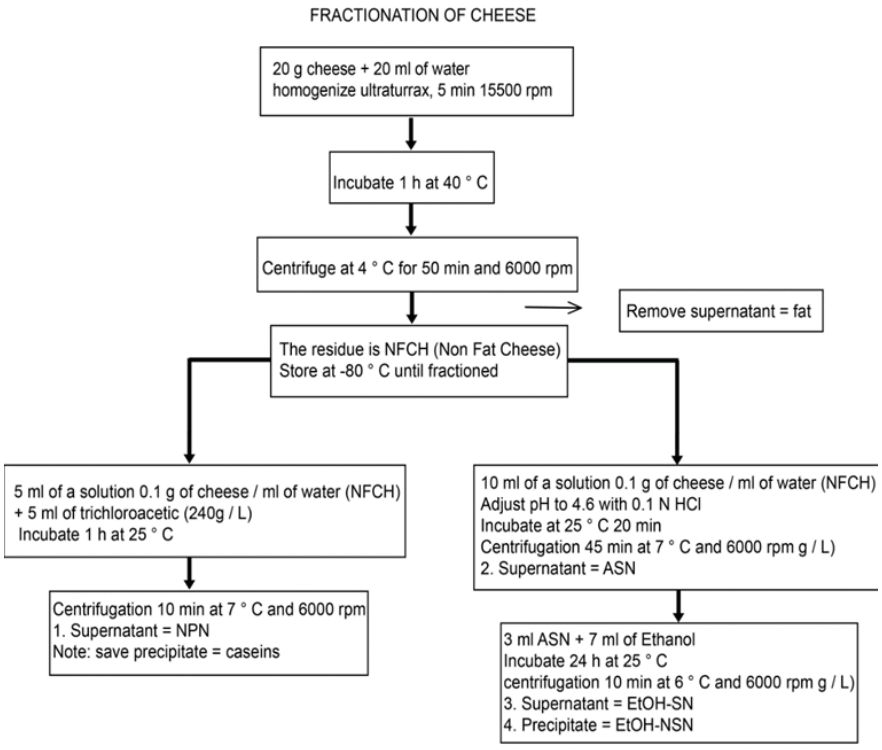

Figure 1. Fractionation of fresh cheese panela type of Queretaro city, México.

Evaluation of the Inhibitory Activity of the Angiotensin-Converting Enzyme (ACE)

To determine the enzyme inhibition in fresh cheese, we used the method of Wang et al. (2013) with some modifications. The method is based on the release of hippuric acid (HA) from hipuril-histidyl-leucine (HHL) catalyzed by the angiotensin-converting enzyme (ACE), the mobile phase consists of 70% A (TriFluoroacetic Acid 0.05% in HPLC water) and 30% B (100% acetonitrile) using a C18 Agilent Technologies column in an HPLC with diode detector, with isocratic flow (70:30 A / B) 0.5 ml/min. The wavelength was 226 nm; the column temperature was controlled at 25°C, a blank with 100% HHL equivalent to 100% ACE was used; HHL and ACE (0.5 U pig kidney) was dissolved in 100 mM borate buffer (pH = 8.3) supplemented with 300 mM NaCl, at a concentration of 0.1 U / ml. 0.025 ml aliquots of the ETOH-SN and ETOH NSN fractions were taken by pre-incubating them with 0.025 ml of ACE at 37°C for 10 min, then 0.025 ml of HHL was added, and it was incubated at 30°C for 30 min. The reaction was stopped by adding 0.0835 ml of 0.1 M HCL. We used as a negative control of functioning enzyme the angiotensin and as positive control the drug captopril, which inhibits the angiotensin-converting enzyme between 90 and 100% of the ACE concentration used (Vargas-León et al. 2018).

ASN and NPN are analyzed directly in the HPLC, for ETOH-SN, 2 ml were concentrated to dryness and then solubilized in 1 ml of 70% ethanol. ETOH NSN precipitate was solubilized in 0.5 ml of 50 µM Tris-HCl and 1 µl EDTA. We filtered the samples with a 0.45 µm syringe filter. Then we used the following formula to calculate the percentage of inhibition:

$$ACEI(\%) = \%HHL - \%HA$$

where:

$$\%HHL = \frac{(\text{Area HHL})}{(\text{Area HHL} + \text{área HA})} * 100$$

$$\%HA = \frac{(\text{Area HA})}{(\text{Area HHL} + \text{área HA})} *100$$

*HHL: Hippuryl-histidyl-leucine.
*HA: Hippuric acid.

Determination of Antioxidant Activity by Reduction of DPPH

The 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical method that is used to evaluate the antioxidant capacity. The reduction of DPPH by antioxidant species

produces a loss of color with a change from intense purple to yellow when the activity is maximum. We used 96 flat-bottom well plates and a standard (BHT, 500 μM) to perform the analysis. All the assays were performed in triplicate. 0.2 mL of DPPH 125 μM DPPH solution in 80% methanol was added. The plate was kept in the dark place for 90 minutes, and then the reading was done in a spectrophotometer at 520 nm. The results were expressed as a percentage of discoloration.

The formula for the calculation of the percentage of discoloration was

$$\% \text{ Of discoloration } = \frac{100* \text{ control} - \text{A sample}}{\text{A control}}$$

A control = absorbance of the control (20 μl methanol + 200 μL)
A sample = Absorbance of the sample or controls (BHT, gallic acid)

Statistical Analysis

All the experiments were performed in triplicates to obtain confinable results. The results of the percentage of inhibition of the ACEI and antioxidant activities were analyzed using free software R version 3.5.2. For the ECEI results, a Kruskal-Wallis nonparametric test was performed, with a confidence percentage of 95%. On the other hand, for the antioxidant activity, a dose-response curve was used to determine the minimum inhibitory concentration.

RESULTS

Based on the evidence, bioactive peptides with the lower molecular weight had a better antioxidant effect; we decided to use ETOH-SN and ETOH NSN because of its content of bioactive peptides with lower molecular weight and discard fractions with ASN, NPN. The fractions with ASN, NPN, according to literature, could contain higher molecular weight peptides. Besides, it was decided only to use the sample one, two, and seven due to the amount of material to perform the analyzes.

ACE Inhibitory Activity

For this part of the study, we determined the inhibitory activity of the ACE in different samples of cheese (A, B), and fractions ETOH-SN, and ETOH-NSN. It was decided to make a comparison between the samples and the control group. We determined in both

samples, inhibitory activity of the angiotensin-converting enzyme (ACE) of the samples, with a significant difference between the control group and the samples with a p = 0.006, Table 3. In contrast, a comparison between the samples (1A, 1B, 2A, 2B, 7A, 7B) of ETOH-NSN presents differences with a value of p = 3.264x10-7 and a percentage range between 49 to 69, Table 3.

The samples of the ETOH-SN fraction did not show differences between them, with a value of p = 0.693. This result suggested that all samples of the ETOH-SN fraction have a similar action effect of ACEI, ranged between 81 and 91%, as shown in tab 3. Sample 1B and 2B showed the highest percentage of inhibition, with 91%. Also, to evaluate the efficacy of the analyzed samples, we compared the percentage of inhibition of the ACE of the cheese samples with the action effect of one of the most used antihypertensive treatments in the pharmaceutical market, captopril with 90%-100% ACEI effect (Vargas-León et al. 2018), being satisfactory because our results showed a similarity in the ACEI effect.

Table 3. The inhibitory activity of the angiotensin-converting enzyme (ACE) of the bioactive peptides from the different cheese samples by high-performance liquid chromatography (HPLC)

ETOH-SN			ETOH-NSN	
Samples		p-value		p-value
1 A	84 ± 3.16	0.693	49 ± 20.27	3.264x10-7
1 B	91 ± 6.72	0.693	65 ± 0.23	3.264x10-7
2 A	86 ± 0.41	0.693	59 ± 7.98	3.264x10-7
2 B	91 ± 6.72	0.693	50 ± 18.75	3.264x10-7
7 A	81 ± 11.03	0.693	63 ± 0.27	3.264x10-7
7 B	90 ± 5.58	0.693	63 ± 0.27	3.264x10-7
Angiotensin control*	4 ± 0.08	0.00656	4 ± 0.08	5,252x10-8

*angiotensin control was performed as an internal control to determinate the inhibition of the samples. Results represent the average of 3 independent experiments ± standard deviation.

Inhibitory Effect of Angiotensin-Converting Enzyme in ETOH-SN vs ETOH-NSN -NSN

After analyzing the ACE inhibitory activity of both fractions, a comparison was made between the ETOH-SN and ETOH-NSN fractions of all the cheese samples. We determinate if the supernatant fraction has a higher inhibitory effect than the non-supernatant. Figure 2 shows that the percentage inhibition of the ETOH-SN group is higher in all the samples analyzed compared to the ETOH-NSN fraction with p-value = 0.02. Due to this, we can infer that ETOH-SN samples have a higher ACE inhibitory, potentially will be recommended not to use ETOH-NSN samples since the percentage inhibition of this fraction is deficient in comparison with the ETOH-SN group.

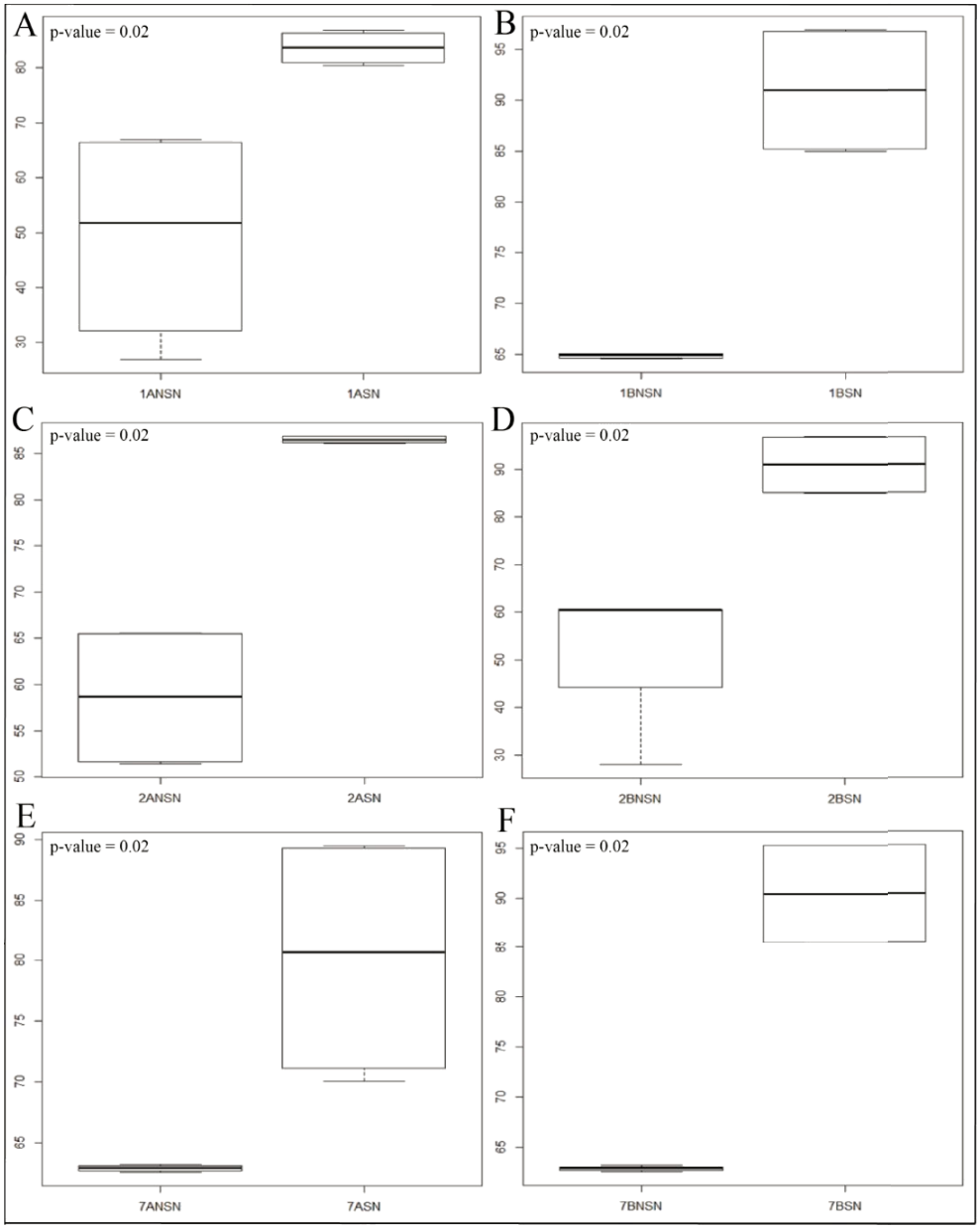

Figure 2. Show the correlation between the supernatant (SN) and precipitate (NSN) of batches. (A) 1 ANSN vs 1ASN, (B) 1 BNSN vs 1BSN, (C) 2 ANSN vs 2ASN, (D) 2 BNSN vs 2BSN, (E) 7 ANSN vs 7ASN and (F) 7 BNSN vs 7BSN. The result is shown in percent of ACE inhibition.

Antioxidant Activity Evaluation

During the chapter, we did determinate that the cheese fractions have an inhibitory effect of ACE, leading to think the possible antioxidant activity of the fraction with higher effect, such as ETOH-SN.

Table 4. Antioxidant activity (DPPH) of the supernatant fraction (ETHO-SN) from the different cheese samples*

	A1	A2	A3	A4	A6
C[1]					
0.01	33 ± 38.72	3 ± 8.66	13 ± 0.43	9 ± 0.74	6 ± 9.42
0.05	17 ± 8.84	6 ± 7.61	39 ± 5.60	10 ± 3.45	9 ± 2.10
0.125	42 ± 4.40	24 ± 8.88	86 ± 0.20	36 ± 1.13	19 ± 5.89
0.25	85 ± 4.95	72 ± 14.6	87 ± 0.47	71 ± 2.86	32 ± 8.79
0.5	107 ± 2.48	92 ± 0.72	86 ± 0.66	83 ± 1.53	71 ± 5.64
1	95 ± 0.68	92 ± 0.13	87 ± 0.66	82 ± 0.00	84 ± 2.21

[1]Concentration is expressed in mg/ml.

Results represent the average of 3 independent experiments ± standard deviation.

We compare the samples A1, A2, A3, A4, A6 in different concentrations, and determinate the DPPH inhibition percentage. As we showed in Table 4, the means and the standard deviation of the DPPH inhibition percentage of the samples of lot A are shown. Each sample was analyzed at different concentrations to determine which of these has a more significant inhibitory effect.

We observed a significant variation in the antioxidant activity of the samples in their different concentrations, where the A3 sample was the one that showed a higher antioxidant effect in most of its concentrations.

Inhibition DPPH

The dose-response curve was performed to determinate the inhibition of DPPH using the inhibitory concentration. The dose-response curve could be able to identify the sample with the higher effect. The inhibitory concentration 50 (IC 50) is the concentration of peptides that inhibits the activity of the DPPH by 50%. Within these, we observe that the sample A3 is the one that presents the best inhibitory potential of DPPH, and we realize a comparative analysis between the means of the samples to obtain p-value with significant difference (0.06) as shown in Table 5 and Figure 3.

Table 5. Antioxidative activity expressed as DPPH inhibition (IC%50) of different samples

Sample	IC50 (M,SD)
A1	0,16 ± 1.07
A2	0,17 ± 1.01
A3	0,06 ± 1.02
A4	0,15 ± 1.02
A6	0,33 ± 1.11

Each sample contains the (mean, m ± standard error, SD) of (n=18).

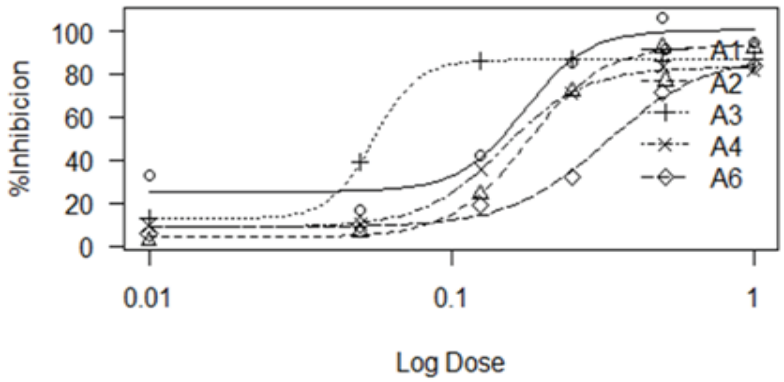

Figure 3. Dose-response of the antioxidant activity (IC%50). We show the samples available of the fraction ETOH-SN. Each curve represents a sample. We determinate the IC50 curve with the Log (DOSE) vs %Inhibition. The figure was made using, R program.

DISCUSSION

During this chapter, we showed some novel results of how cheese, especially cheese fractions, can have a high activity such as an inhibition of ECA and antioxidant. Initially, the fractionation of the samples was performed using proteolysis as a method (Guerra-Martínez, Montejano, and Martín-Del-Campo 2012), once the fractions were obtained, they were called supernatant (ETHO-SN) and precipitate (ETHO-NSN). Interestingly, our results lead us to determinate that the supernatant is the fraction which has more effective results, related to other studies (Qian et al. 2011; Aguilar-Toala et al. 2017).

The ACE inhibitory capacity of the different fractions was determined, it was observed that both fractions had a notable inhibitory effect compared to the control group with a p-value of 0.006 in ETOH-SN and $5.252x10^{-8}$ in ETOH-NSN. Also, we determinate that the supernatant fraction had a significative inhibitory effect compared to the precipitated fraction with a p-value of 0.02. This result is based on the fact that the hydrolyzed fractions with lower molecular weight contain bioactive peptides with higher effect (Qian et al. 2011; Aguilar-Toala et al. 2017). It is essential to clarify that this fraction (ETOH-SN) was chosen to measure antioxidant activity because it was the fraction that had the most significant ACE inhibitory effect, but not because it is the fraction that has the highest number of peptides. The evaluation of antioxidant activity in this fraction is justified by the references described in the presence of antioxidant peptides present in cheeses (Chel-Guerrero et al. 2009; Aguilar-Toala 2014). We will considered the determination of IC50 in further studies to understand better the antioxidant activity. Also, we observe that among the samples of the ETOH-SN fraction, there is no significant difference with a value of p = 3,264x10-7, so we could catalog all samples with good ACEI effect and good antioxidant activity.

To determine the effect of our study, we collect information from different researches (Table 6), generating a comprehensive review of original studies published from 2003 to 2018 in science direct, PubMed, and google scholar. The search was performed on February 8th, 2019, using the terms "bioactive peptides in milk products" "ACE," "ACEI," "cheese," "antioxidant" with the filter "publications date" 15 years. Three authors (KG, GM, and JM) reviewed the abstract, and standard criteria were used to exclude the following publications, with the exclusion criteria "non-lacteous product" and different methodology leaving 14 references to analyze. All the authors perform an abstract reading focusing on original articles and the antioxidant activity "DPPH" or ACEI activity. The Table we divided into different types of Cheese according to the principal activity of interest such as IC50 IECA and DPPH antioxidant. Therefore, we included a comment on the relevance of the paper and the references. In Table 6, we obtained 41 kinds of cheese in which included fresh, panela, Bria, cheddar, blue cheeses. The cheeses analyzed had the antioxidant activity and IECA activity similar to our results.

Most of the cheeses have an inhibitory effect of ACE with values such as 26-96%, with an average of 61%. Comparing these percentages with the percentage of our results (90%) we can infer that our analyzed samples have a high inhibitory ECA effect. In addition to the inhibitory effect of captopril, the examples of the ETOH-SN fraction are similar, having the possibility of being considered as a cardioprotective treatment.

Considering the results of the ACEI analyzed and shown in Figure 2, it was decided to work with the supernatant fraction, to identify the antioxidant activity of the samples, so we could evaluate if the fraction has the highest content of bioactive peptides. As we shown in results, the DPPH free radical method is used for its rapid and direct reaction with antioxidant compounds, facilitating the identification of the antioxidant power of the samples (Wongmekiat, Thamprasert, and LumLertgul 2007).

To evaluate the antioxidant effect, we took information from Table 6. We observed a fluctuation between 30 and 80% of the DPPH inhibition percentage of samples of different types of cheeses, in comparison to result where there was a variation that ranged from 6 to 107%, where the A3 sample showed a more significant antioxidant effect in most of the concentrations analyzed. After this, to corroborate which sample had the most significant impact, a dose-response curve was performed, with the A3 example showing the highest antioxidant activity with an average IC-50 of 0.06 (mL/dl).

All these results are due to the role played by microorganisms in the proteolysis of cheese peptides, producing bioactive peptides (milk-derived multifunctional peptides, lactoferrin-derived peptides, casein-derived phosphopeptides, and chymotryptic peptides) obtained from the fermentation with *Lactobacillus plantarum* strain showed in this research (Fitzgerald and Murray 2006; Aguilar-Toala et al. 2017), and therefore we can find a high content of bioactive peptides even in fresh cheeses traditionally made (Li et al. 2011).

Table 6. Analysis and bibliographic review

Sample		Methods		Comments	Reference
		ACEI (%)* IC†	DPPH (%)		
1	Manchego cheese	93.5 ± 1.4*	NA	These cheese produce peptides that inhibit ACE and can be used as cardioprotector.	Gómez-Ruiz, Ramos, and Recio 2002
2	Mexican fresco cheese	96*	NA	Mexican cheese manufactured with specific LAB strains produces peptides with potential health benefits, like ACE inhibitors	Torres-Llanez et al. 2011
3	Milk fermented with *L. lactis*	74-98*	NA	Products derived from milk have the presence of peptides with antihypertensive activity especially those that are enriched with microorganism as *L. lactis*	Rodríguez-Figueroa et al. 2010
4	Gamalost	1.0 ± 0.2+	NA	Gamalost with a high degree of proteolysis and high protein content seems to have a high ACE inhibitor. Traditional cheese had per unit cheese weight higher ace inhibitor	Pripp et al. 2006
5	Castello	1.1 ± 0.2+			
6	Brie	1.3 ± 0.3+			
7	Pultost	1.3 ± 0.5+			
8	Norvegia (9months)	1.7 ± 0.6+			
9	Port Salut	1.4 ± 0.1+		potential than the rest of cheese type.	
10	Norvegia	1.6 ± 0.2+			
11	Kesam	7.7 ± 1.4+			
12	CP Cabot plain (cheddar)	74*	33	The highest ace inhibitors activity was shown in plain and herb enriched cheddar cheese as well as cranberry enriched cheese.	Apostolidis, Kwon, and Shetty 2007
13	CH Cabot herbs (cheddar)	73*	58		
14	FP Athenos plain (feta)	71*	26		
15	FH Athenos herbs (feta)	69*	65		
16	R1 Pupilon Roquefort	28*	89		
17	R2 Rosenborg Roquefort	39*	90		
18	R3 Blue stillon (Roquefort)	26*	87		
19	Wensleydale (English and hard cheese) WC	71*	30		
20	Cheddar cheese	NA	0.025% = 47.76 0.05% = 53.34 0.075% = 56.32 0.1% = 68.81	The antioxidant activity in cheddar type cheeses increase with the fortification with IBE	Lee et al. 2015
21	A-Feta type, Brazil	46*	NA	Among the different types of cheeses analyzed, Roquefort type cheese demonstrated a better set of bioactive.	Segalin et al. 2012
22	A- Pecorino Toscano-Type, Brazil	60days = 72* 180days = 69* 270days = 62*			
23	B-Feta-Type, Brazil	60days=74*		Among the different types of cheeses analyzed, Roquefort type cheese demonstrated a better set of bioactivities.	Segalin et al. 2012
24	B- Roquefort Type, Brazil	90days = 80*			

Sample		Methods		Comments	Reference
		ACEI (%)* IC†	DPPH (%)		
25	C- Pecorino-type Brazil	30days = 73*			
26	Pecorino Sardo-type, Uruguay	80days = 70* 120days = 69* 160days = 56*	NA		
27	Cerillano, Uruguay	90days = 64* 120days = 75*			
28	Italico cheese	Fraction26 = 82*	NA	Cheeses and milk products are a potential source of bioactive peptides with a multifunctional role; the proteolysis may be conditioned by inhibitory peptides generated during manufacturing and cheese ripening.	Smacchi and Gobbetti 1998
29	Gorgonzola cheese	Fraction 5 = 80*			
30	Idiazabal Cheese	87.5 ± 2.5*	NA	The study shows the presence of ace inhibitory activity in different Spanish cheese made with different technologies. We found that the activity was primarily concentrated in the permeate <1000Da.	Gómez-Ruiz et al. 2006
31	Roncal cheese	85.8 ± 0.5*			
32	Mahón cheese	76.8 ± 5.6*			
33	Goat cheese	72.8 ± 7.7*	NA	The study shows the presence of ace inhibitory activity in different Spanish cheese made with different technologies. We found that the activity was primarily concentrated in the permeate <1000Da	Gómez-Ruiz et al. 2006
34	Cabrales	74.7 ± 2.1*			
35	Manchego	70.6 ± 1.5*			
36	Burgos type cheese 1	NA	1mg/mL =5.17 ± 2.60 3mg/mL =22.64 ± 3.32 6mg/mL =50.64 ± 9.87 9mg/mL =72.08 ± 4.12 12mg/mL=77.67 ± 10.03	Three peptides (derived from casein) were identified from Burgos type cheese, the cheese manufactured with rennet of plant origin contained the highest proportion of these peptides. Peptides derived from casein have already been described as antioxidant peptides.	Petrón et al. 2014
37	Burgos type cheese 2		1mg/mL =5.17 ± 2.60 3mg/mL =22.64 ± 3.32 6mg/mL =50.64 ± 9.87 9mg/mL =72.08 ± 4.12 12mg/mL=77.67 ± 10.03		
38	Burgos type cheese 3		1mg/mL =8.99 ± 2.18 3mg/mL =24.85 ± 5.57 6mg/mL =50.16 ± 11.35 9mg/mL =71.08 ± 11.14 12mg/mL =82.16 ± 3.29		
39	Cheddar cheese 1	0.06^{+} 0.08^{+} 0.30^{+}	8 11 13	The preliminary result of this study shows that all three cheddar cheese peptide extracts exhibit bioactivity, the vintage cheese taught the most potent inhibitory activity of ACE.	Pritchard, Phillips, and Kailasapath 2010
40	Cheddar cheese 2	0.11^{+} 0.23^{+} 0.14^{+}	12 12 11		
41	Cheddar cheese 3	0.14^{+} 0.7^{+} 0.13^{+}	10 11 12		

* Percentage of ACEI, † Half-maximal inhibitory concentration (IC_{50}).

Not available (NA).

It has been shown that products containing different biologically active components are associated with nutrition and health benefits (Sah et al. 2014; Ebringer, Ferencik, and Krajcovic 1986). Previous research has helped to locate dairy products as foods with a possible cardioprotective effect (Pripp et al. 2006; Pripp 2008), this property has been attributed to several factors, but now it is known that it is due to the formation of peptides with antihypertensive and antioxidant activity (Smacchi and Gobbetti 1998).

Additionally, in a study conducted by Tohoku University in Japan, working with SnowBrand Milk Products Co., Ltd., they proved that cheese whey proteins could have beneficial prevention and biological and nutritional health promotion (Erdmann, Cheung and Schröder 2008). The amino acid present in this protein, such as tyrosine and cysteine, may exert free radical scavenging activity (Korhonen and Pihlanto 2006).

Finally, to justify these results, it is suggested to evaluate cheese fractions through an experimental in vivo study, where the effect of panela cheese consumption in patients with hypertension or cardiovascular diseases will be evaluated. Other studies should be performed, integrating the possibility of using this food as an alternative treatment for a risk group and considering different factors such as the frequency of consumption, the type of cheese, and the availability of people to acquire it. Besides, we recommend carrying out studies on fresh cheeses and other Ecuadorian dairy products, to identify the possible antihypertensive and antioxidant properties that we have mention that exists.

CONCLUSION

The samples of Mexican panela cheese analyzed in this study showed the inhibitory activity of ACE and antioxidant, especially ETOH-SN that was the one that proved to have a more significant effect of action, with a p-value of 0.02 we showed a significant difference in comparison to the other fraction analyzed.

Our study indicates that the fraction with the lowest molecular weight ETOH-SN showed a better antioxidant and ACEI effect, being able to attribute these results to the fact that in this fraction, there is a higher amount of bioactive peptides with a more significant impact of the action.

With all this evidence, we can infer that the consumption of these peptides might be considered a valuable tool against different human diseases, so including panela-type cheeses in the diet could be a good alternative for patients with cardiovascular treatments, such as hypertension.

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