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Comparison of microwave-, ultrasound- and enzyme-assisted hydrolysis for producing antioxidant whey protein hydrolysates

Comparación de la hidrólisis asistida por microondas, ultrasonido y enzimas para la producción de hidrolizados antioxidantes de proteína de suero

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Abstract

Whey from asadero cheese, a byproduct with a high environmental impact (~9 L per kg of cheese), represents a strategic raw material for obtaining bioactive peptides under a circular economy approach. This study evaluated the generation of hydrolysates and their antioxidant potential using primarily microwave-assisted hydrolysis (600 W with a ramp to 60 and 80°C, and 15 min isothermal; and non-ramp assays comparing 600 and 900 W), as well as ultrasound (40 kHz; 15 min), and enzymatic hydrolysis (*Aspergillus niger* M4 extract; 35°C; 15 min) as reference treatments. After the treatments, the <50 kDa fraction was recovered, and relative TCA-soluble peptides content, expressed as tyrosine equivalents as an indirect index, and antioxidant capacity were measured by DPPH and ABTS reduction. The enzymatic treatment yielded the highest peptide concentration under mild conditions (T5: 1.28 mg Tyr eq/mL) with a 35% degree of hydrolysis, followed by microwave treatment at 600 W and 60 °C (T1: 1.19 mg Tyr eq/mL). The best antioxidant activity was observed with the microwave method at 900 W without a ramp, reaching 160 °C (T7), which achieved 71.5% DPPH inhibition. The results suggest, based on indirect evidence, that antioxidant capacity may be more strongly associated with the characteristics of the released peptide fraction than with its total content estimated as tyrosine equivalents.

Keywords: protein hydrolysates, enzymatic hydrolysis, microwaves, ultrasound, peptides.

Resumen

El suero de queso asadero, un subproducto con un alto impacto ambiental (~9 L por kg de queso), representa una materia prima para la obtención de péptidos bioactivos bajo un enfoque de economía circular. Este estudio evaluó la generación de hidrolizados y su potencial antioxidante utilizando hidrólisis asistida por microondas (600 W con rampa a 60 y 80 °C, y 15 min isotérmica; y sin rampa 600 y 900 W), así como ultrasonido (40 kHz; 15 min) e hidrólisis enzimática (extracto de *Aspergillus niger* 35 °C; 15 min). Se recuperó la fracción <50 kDa y se cuantificaron los péptidos relativos solubles en TCA como índice indirecto, y la capacidad antioxidante mediante reducción de DPPH y ABTS. El tratamiento enzimático produjo la mayor concentración de péptidos en condiciones suaves (T5: 1.28 mg Tyr eq/mL) con un grado de hidrólisis del 35 %, seguido del tratamiento con microondas a 600 W y 60 °C (T1: 1.19 mg Tyr eq/mL). La mejor actividad antioxidante se observó con el método de microondas a 900 W sin rampa, alcanzando 160 °C (T7), con una inhibición DPPH del 71.5 %. Los resultados sugieren, en base a evidencia indirecta, que la capacidad antioxidante puede ser fuertemente asociada con las características de las fracciones de los péptidos liberados, que por su cantidad total estimada como equivalentes de tirosina.

Palabras clave: digestión anaeróbica, estiércol de vaca, ácido giberélico, ácido indolacético, fitorreguladores.

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1 Introduction

Whey is commonly managed as a byproduct in the dairy industry. Its untreated discharge into drainage systems increases the pollutant load and environmental risk. This underutilization is particularly relevant because whey retains valuable amounts of proteins, lactose, and mineral salts, which makes it an attractive substrate for biotechnological revalorization (Patlan-Velazquez *et al.*, 2025). The production of 1 kg of fresh asadero cheese generates around 9 L of waste whey (Fernández-Gutiérrez *et al.*, 2017; Sebastián-Nicolás *et al.*, 2020). Whey contains proteins that can be degraded by hydrolysis, generating peptides that typically contain 3 to 20 amino acids per peptide, with fractions smaller than 50 kDa (Sebastián-Nicolás *et al.*, 2020). Peptides generated by whey protein hydrolysis are considered bioactive peptides, which are amino acid sequences that provide biological functions beyond their nutritional value (Morgan & Breen, 2021; Prokopidis *et al.*, 2025). Among the main reported bioactivities of food-derived bioactive peptides is the attenuation of cellular oxidative stress, including reduced ROS generation and protection against oxidant-induced damage (Tonolo *et al.*, 2018), a property that may support future exploration in agriculture, food and health sectors.

In current agricultural and horticultural systems, strategies based on biostimulants capable of improving nutritional efficiency, crop quality, and tolerance to abiotic stress have gained interest. In this context, protein hydrolysates (mixtures of peptides and amino acids), or PH, are considered a relevant category of biostimulants due to their positive effects on crop performance (Colla *et al.*, 2015). Abiotic stress increases the generation of reactive oxygen species (ROS) and oxidative stress, affecting crop growth, yield, and quality (Qamer *et al.*, 2021). Within this framework, PH containing antioxidant peptides are emerging as sustainable biostimulants due to their potential to modulate antioxidant responses. Peptides derived from the hydrolysis of whey protein have been reported to stimulate tolerance to abiotic stress and promote plant growth (Alfonso *et al.*, 2025). Several studies have shown that the application of PH via foliar and root application improves N and Fe metabolism, as well as nutrient and water uptake efficiency, associated with changes in root architecture and key enzymes of nitrogen metabolism (Colla *et al.*, 2015).

Protein hydrolysis can be achieved through various methods, the most common being those employing an acid or base. Other alternative methods, such as enzymatic hydrolysis, microwave-assisted processes, and ultrasound-assisted processes, allow for the optimization of bioactive peptide production without the use of chemical solvents (Sharma *et al.*, 2023). Each method operates through different mechanisms on the protein matrix, which could modify the degree of hydrolysis, the size distribution of the released peptides, and consequently, the bioactivity of the resulting hydrolysates.

Enzymatic hydrolysis catalyzes the breaking of peptide bonds, generating complex mixtures of fragments of varying lengths, which can coexist with partially hydrolyzed protein fractions (Beaubier *et al.*, 2023). It is one of the most widely used methods due to its specificity and the possibility of controlling conditions that favor functional properties. Commonly used enzymes include proteases such as trypsin or pepsin to obtain smaller peptides (Gasparre *et al.*, 2025; Londoño-Hernández *et al.*, 2024). In a study by Sakkas *et al.* (2022), it was reported that the action of trypsin on milk proteins can generate peptides with antioxidant and even antihypertensive activity. The formation of peptides with antimicrobial function has also been investigated, with applications in functional foods and potentially in the manufacture of agricultural biostimulants (Zhu *et al.*, 2022).

Microwave-assisted hydrolysis, on the other hand, is a non-conventional technology that can accelerate the process by promoting rapid and homogeneous heating of the system. The high energy of microwaves increases the reaction rate and facilitates peptide bond cleavage, influencing the yield and functional properties of the hydrolysate (El Mecherfi *et al.*, 2019). Several studies have indicated improvements in functional properties and antioxidant capacity associated with hydrolysates obtained under microwave-assisted hydrolysis (Katherine *et al.*, 2023; Noman *et al.*, 2020). Reported operating conditions include a power range of 0–1000 W and temperatures of 37–70°C for milk and whey proteins, with evidence of protein degradation and peptide formation (El Mecherfi *et al.*, 2019; Yang *et al.*, 2022). In contrast, ultrasound-assisted hydrolysis uses high-frequency acoustic waves that induce cavitation, which breaks down protein structures and promotes the rate of hydrolysis, maintaining the integrity of bioactive peptides (Habinshuti *et al.*, 2021; Taha *et al.*, 2024; Yolandani *et al.*, 2023; Zhang *et al.*, 2024). In dairy matrices, the effect of ultrasound is strongly associated with acoustic cavitation, and when combined with moderate heating, cavitation efficiency may increase, promoting greater molecular disruption and modifications in functional properties while reducing severe thermal damage (Monter-Arciniega *et al.*, 2025). Recent studies commonly use ultrasound times on the order of 10–40 min and power inputs of 200–800 W, depending on the protein system and equipment configuration (Pang *et al.*, 2024; Qian *et al.*, 2023; Shokri *et al.*, 2022). Despite the progress

in these technologies, it remains necessary to compare and further investigate their application to different substrates, and to relate the process conditions to the peptide content and the resulting antioxidant activity (Habinshuti *et al.*, 2021).

Despite the progress in alternative hydrolysis technologies, comparative studies using the same whey matrix and relating processing conditions to peptide release and antioxidant activity are still limited. We hypothesized that microwave, ultrasound, and enzymatic treatments would produce hydrolysates with different peptide contents and antioxidant responses because of their distinct mechanisms of action on whey proteins. Therefore, this study provides a direct comparison of these three hydrolysis strategies in asadero cheese whey, linking degree of hydrolysis and relative TCA-soluble peptide content estimated by tyrosine equivalents in the <50 kDa fraction with DPPH and ABTS antioxidant activity as a contribution to whey revalorization.

2 Materials and methods

2.1 Raw material collection

This research was conducted at the Fermentation and Biomolecules Laboratory of the Universidad Autónoma Agraria Antonio Narro, in collaboration with the Biorefinery group of the Universidad Autónoma de Coahuila. Whey was collected from a cheese factory in Saltillo, Coahuila, Mexico, as a byproduct of the production of "queso asadero" (Figure 1) from cow's milk sourced from small farms in surrounding communities. The liquid material was stored in glass bottles under refrigeration (3-5 °C) for 24 h until use.

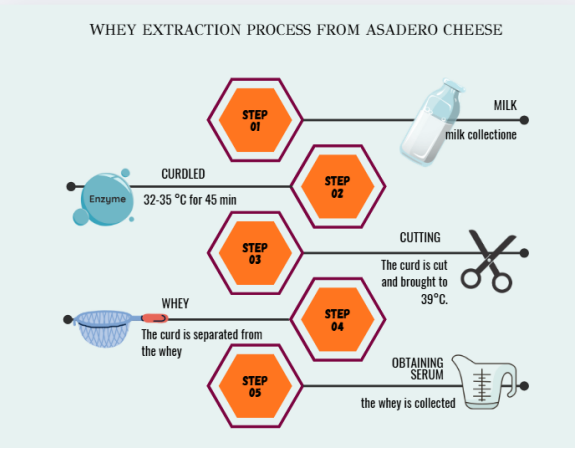


Figure 1. Scheme of the whey process during the making of asadero cheese: milk collection, enzymatic coagulation (32–35 °C, 45 min), cutting and heating of the curd (39 °C), curd-whey separation, and whey collection.

Proximate characterization of the whey showed a protein content of 9.58% (Marín-Cortez *et al.*, 2025).

2.2 Methods and conditions of hydrolysis

Microwave-assisted hydrolysis: For this method, a CEM Mars 6 microwave (~2450 MHz) was used, with a working volume of 50 mL of whey and four replicates. A power of 600 W was used as an intermediate condition to ensure rapid volumetric heating without increasing the severity that could induce browning, aggregation, or degradation of the peptides. Hydrolysis temperatures were 60 and 80 °C, with a linear temperature progression until the hydrolysis temperature was reached (Table 1). The 60 °C temperature, as a moderate condition, favors partial denaturation with a lower risk of degradation, while the 80 °C temperature increased susceptibility to breakdown and the degree of denaturation (Hettiarachchi *et al.*, 2012; Meng *et al.*, 2026). Once the hydrolysis temperature was reached, a 15-minute isothermal phase was maintained. In an additional assay, a comparison of 600 and 900 W power levels was made without a linear temperature progression, in order to evaluate the direct effect of the energy input and the characteristic heating rate of microwaves. To improve comparability, all treatments were conducted using the same whey matrix, a working volume of 50 mL, a treatment time of 15 min, and identical downstream analytical procedures. Nevertheless, because microwave, ultrasound, and enzymatic hydrolysis differ in their mechanisms of action and thermal severity, the comparison should be understood as a standardized process comparison and not as a strict energy-matched evaluation.

Ultrasound-assisted hydrolysis and temperature: A Cole-Parmer UC 300 Series ultrasound system was used, with a volume of 50 mL of whey and four repetitions. The system operated at 600 W nominal power at 40 kHz at 100% amplitude. Temperature during sonication was controlled using a jacketed hydrolysis vessel, through which running water was continuously circulated to dissipate the generated heat and maintain the hydrolysis temperature for 15 min, as indicated in the literature (Carrillo-Lopez *et al.*, 2021; Resendiz-Vazquez *et al.*, 2017).

Enzyme-assisted hydrolysis with extract: For the enzymatic hydrolysis treatment, the enzyme extract (EE) was obtained according to the methodology of Marín-Cortez *et al.* (2025). For the hydrolysis process, 50 mL of whey and 2.70 mL of EE (specific activity of 1.51 U/mg) were used in four replicates. The hydrolysis mixture (EE + whey) was left to react for 15 min at a temperature of 35 °C under isothermal conditions.

Table 1. Experimental conditions of hydrolysis treatments applied to whey (microwave, ultrasound and enzymatic): method, final temperature and processing time.

Treatment	Method	Final temperature (°C)	Time (min)
T1	Microwave 600 W	60	15+6*
T2	Microwave 600 W	80	15+8*
T3	Ultrasound 40 kHz	60	15
T4	Ultrasound 40 kHz	80	15
T5	Enzymatic	35	15

* In microwave treatments, the time is reported as 15 min isothermal stage + ramp time to reach target temperature (6 min at 60 °C; 8 min at 80 °C).

2.3 Determination of soluble proteins and peptides

Hydrolysis progress was assessed by quantifying relative TCA-soluble peptide content and the degree of hydrolysis (DH) (Czelej *et al.*, 2025). The hydrolyzed extracts were centrifuged (Hermle laborTechnik, model Z 32 HK) at 4000 rpm for 15 min at 4°C, and the supernatant was recovered. The supernatants were filtered by centrifugation (4000 rpm, 20 min, and 4°C) using Amicon® Ultra-4 filters in two stages. The first stage used 100 kDa filters, followed by 50 kDa filters. The permeate fraction was mixed with a 1:1 volume of 5% (v/v) TCA and incubated for 15 min at room temperature. The sample was centrifuged at 4000 rpm for 15 min at 4°C, and the supernatant was considered the concentrated peptide extract (CPE) corresponding to TCA-soluble compounds with an apparent molecular weight less than 50 kDa. The <50 kDa cutoff was used as an operational fractionation criterion to remove residual higher-molecular-weight proteins and aggregates, while recovering a soluble hydrolysate fraction under the same downstream conditions for all treatments. Thus, this fraction was selected for comparative purposes and should not be interpreted as a purified fraction of low-molecular-weight bioactive peptides.

Relative TCA-soluble peptide content in the CPE was estimated by UV absorbance at 280 nm (Thermo Fisher Scientific, model G10S) with a 1:10 dilution. The results were expressed as tyrosine equivalents (mg Tyr eq/mL), using a calibration curve prepared with L-tyrosine (FAGALab) in 1 M HCl (Jalmek, Mexico) in the range of 0–0.156 mg/mL ($y = 0.0028x + 0.0011$; $R^2 = 0.9998$). Absorbance at 280 nm depends primarily on aromatic residues (tyrosine/tryptophan). This measurement represents a relative index of TCA-soluble peptide/aromatic compounds (Wang *et al.*, 2019). Therefore, this measurement may underestimate peptides lacking aromatic amino acids and does not distinguish between free tyrosine and tyrosine present in peptides. To estimate the DH under the present analytical conditions, relative TCA-soluble peptides were quantified in the mixtures of each treatment and in untreated whey, according to the following formula:

$$DH\ (\%) = \frac{P_t - P_0}{P_{tot}} * 100 \quad (1)$$

Where P_t is the TCA-soluble compounds in the hydrolyzed sample after treatment (mg Tyr eq/mL), P_0 is the concentration of TCA-soluble compounds in untreated whey before hydrolysis (mg Tyr eq/mL), and P_{tot} is the total protein concentration of the original whey matrix (mg/mL). Under these analytical conditions, DH represents an estimate of hydrolysis based on the relative increase in TCA-soluble material after treatment.

2.4 Antioxidant capacity determination

Antioxidant capacity was determined in triplicate using the DPPH assay, following the methodology of Brand-Williams *et al.* (1995), complemented by the ABTS assay, following the methodology of Re *et al.* (1999). A 0.1 mM DPPH radical solution (Aldrich) was prepared in 80% methanol (Jalmek). For each determination, 0.025 mL of sample was mixed with 0.200 mL of the DPPH solution and incubated for 30 min in the dark. Absorbance was then measured at 517 nm using a microplate reader (BIOBASE ELISA BK-EL10A). The results were expressed as the percentage reduction of the DPPH radical, calculated using the equation:

$$DPPH\ Inhibition\ \% = \left[\frac{(A_c - A_m)}{A_c}\right] * 100 \quad (2)$$

Where A_c is the DPPH control absorbance and A_m is the sample absorbance.

For the ABTS assay, a stock solution of ABTS (7 mM) in distilled water and a potassium persulfate solution (2.45 mM) were prepared. Both solutions were mixed in equal volumes and incubated in the dark for 16 h at room temperature to generate the $ABTS^{?+}$ radical solution. Prior to analysis, the radical solution was diluted with a phosphate buffer solution (NaCl 8 g/L, KCl 0.2 g/L, KH_2PO_4 1.44 g/L) (Jalmek) until an absorbance of 0.7 ± 0.02 at 750 nm was reached, as verified using a microplate reader. The determinations were performed in triplicate, mixing

0.10 mL of sample with 0.990 mL of diluted ABTS, at an absorbance of 750 nm in a microplate reader (BIOBASE ELISA BK-EL10A). The results were expressed as a percentage of ABTS radical inhibition, calculated using the equation:

$$ABTS\ Inhibition\ \% = \left[\frac{(A_c - A_m)}{A_c}\right] * 100 \quad (3)$$

Where A_c is the ABTS control absorbance and A_m is the sample absorbance.

2.5 *Statistical analysis*

Arithmetic means of the collected data from the treatments ± their standard deviation are reported (n = 4 for hydrolysis, and n = 3 for analysis). Experimental design and statistical analyses were performed using Minitab 19 statistical software. Assumption tests were considered significant with a p-value > 0.05. A one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple-comparison test ($p < 0.05$) was used to compare the different hydrolysis methods for each parameter (soluble peptides, and ABTS and DPPH antioxidant activity).

3 Results and discussion

Relative TCA-soluble peptide content was used as a comparative indicator of hydrolysis performance among treatments. Figure 2 shows the concentration of relative TCA-soluble peptides, expressed as tyrosine equivalents (mg Tyr eq/mL), obtained by the hydrolysis treatments (microwave, ultrasound, and enzymatic). Treatment T5 (enzymatic) showed the highest concentration (1.28 mg Tyr eq/mL), with no statistically significant difference compared to treatment T1 (microwave 600 W, 60 °C), T2 (microwave 600 W, 80 °C), and T4 (ultrasound 600 W, 80 °C). Similarly, the degree of hydrolysis for T5 was the highest (35.3%), followed by T1 (26.0%), with no statistically significant difference. In the case of the enzymatic treatment, its effectiveness lies in the catalytic and controlled cleavage of peptide bonds, generating more short fragments that remain in the soluble fraction. The enzyme-treated extract of *A. niger* M4 exhibited highly efficient hydrolysis under mild temperature conditions, resulting in greater control of the peptide profile. In the case of treatment T1 at 60 °C with microwaves, the proteins may have been partially unfolded, increasing their solubility without strong aggregation, approaching the levels seen in T5. In contrast, at temperatures of 70–75 °C or higher, whey proteins tend to denature and aggregate due to the exposure of hydrophobic groups and disulfide exchange reactions (Freire *et al.*, 2022; Li

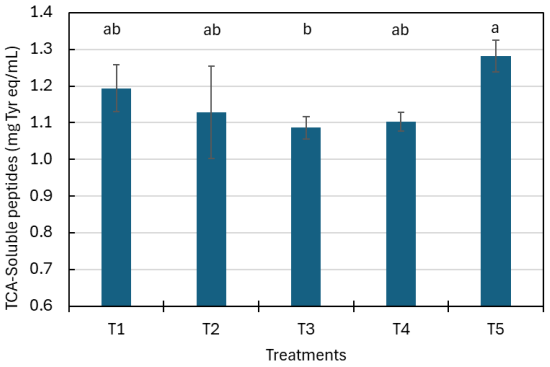
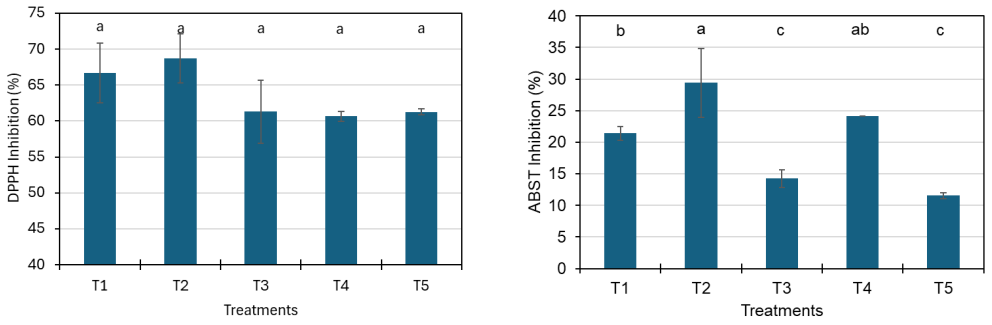


Figure 2. Effect of hydrolysis treatments on peptides. T1: Microwave 60 °C, T2: Microwave 80 °C, T3: Ultrasound 60 °C, T4: Ultrasound 80 °C, T5: Enzyme extract 35 °C. Bars are the arithmetic means of 4 replicates ± the corresponding standard deviation. Means that do not share a letter are significantly different (Tukey $p < 0.05$).

et al., 2021). These chemical aggregations can reduce the soluble fraction recovered after centrifugation and ultrafiltration, explaining why the microwave treatment at 80 °C (T2) did not increase the peptide concentration compared to T1. Temperatures above 80 °C have been reported to favor non-specific structural aggregation, which may modify peptide conformation and the exposure of residues associated with antioxidant activity (Freire *et al.*, 2022; Meng *et al.*, 2026; Zhang *et al.*, 2024). It is worth noting that the quantification of peptides using UV-A280 in tyrosine equivalents depends on the content of TCA-soluble aromatic residues, making it a comparative index rather than an absolute concentration of total peptides.

The antioxidant potential of bioactive peptides from whey protein hydrolysates is a significant parameter due to its link with the reduction of oxidative stress. In this study, the relative soluble TCA fraction was evaluated using DPPH and ABTS radical scavenging assays as complementary indicators of reducing capacity. The assay as a whole allowed for a robust evaluation of the antioxidant potential of relative soluble TCA peptides, strengthening the comparison between treatments (Figure 3). Within this treatment set, T2 showed the highest combined antioxidant response, with 68.7% DPPH inhibition and 29.0% ABTS inhibition. Nevertheless, no statistically significant differences were observed relative to some other treatments, depending on the assay considered. However, treatment performance depended on the analytical criterion considered. While T2 showed the highest antioxidant response within this treatment set, T5 exhibited the highest relative TCA-soluble content and degree of hydrolysis, indicating that greater release of soluble material did not necessarily translate into greater radical scavenging



(a) (b)

Figure 3. Effect of hydrolysis treatments on the percentage of (a) DPPH and (b) ABTS reduction capacity. T1: Microwave 60 °C, T2: Microwave 80 °C, T3: Ultrasound 60 °C, T4: Ultrasound 80 °C, T5: Enzyme extract 35 °C. Bars are the arithmetic means of 3 replicates ± the corresponding standard deviation. Means that do not share a letter are significantly different (Tukey $p < 0.05$).

capacity. These results suggest that the antioxidant response was associated not only with the relative amount of TCA-soluble compounds released, but also with the characteristics of the fraction generated under each hydrolysis condition. Therefore, in this study, "best performance" was defined according to the response variable evaluated: T5 for relative TCA-soluble content and DH, T2 for the highest combined antioxidant response within the isothermal treatments (T1–T5).

In whey proteins, temperatures above 70 °C cause very high denaturation in short periods (minutes), exposing hydrophobic groups and thiols, thus increasing the accessibility of segments with reducing potential (Asaduzzaman *et al.*, 2021; Gao *et al.*, 2018). García-Moreno *et al.* (2023) indicate that different hydrolysis treatments influence the release of hydrophobic and aromatic peptides, increasing DPPH reduction. Some peptides present in whey can exhibit free radical scavenging properties through hydrophobic terminal amino acids, such as Ala (A), Pro (P), Val (V), Ile (I), Leu (L), Phe (F), Trp (W), Tyr (Y), and Met (M) (Melnikova *et al.*, 2022). Another phenomenon that could increase the inhibition of both radicals in the 80 °C microwave system is peptide-sugar glycation reactions. Microwave heating has been reported to accelerate the progression of these reactions and increase antioxidant activity in protein-sugar systems, contributing to greater inhibition in both assays (Nasrollahzadeh *et al.*, 2017; Nooshkam & Madadlou, 2016). The superiority of T2 over T1 (60 °C) is related to the 60 °C temperature, which is below the denaturation threshold of whey proteins, while 80 °C favors deeper transformations. This suggests that increasing temperatures promotes the generation of relative soluble TCA peptides with antioxidant activity.

On the other hand, changing the temperature with ultrasound to 60 °C and 80 °C showed no difference in DPPH radical inhibition, but did show a significantly better response in ABTS inhibition. The greater performance of ABTS at 80 °C with ultrasound may be due to protein unfolding, which, combined with the mechanical action of ultrasound, increases the fraction of more polar or easily reactive compounds in aqueous media (Gao *et al.*, 2018; Vernès *et al.*, 2020). This is consistent with the fact that the ABTS method responds to hydrophilic and lipophilic antioxidants, while DPPH is more sensitive to hydrophobic compounds.

Treatment T5 (enzymatic hydrolysate) showed the highest amount of relative soluble TCA peptides compared to the other treatments. However, these hydrolysates did not show significant antioxidant performance in ABTS and DPPH assays. These results show that the quantity of peptides produced is not synonymous with stronger antioxidant content but rather depends on the characteristics of the peptides and their functional groups. Although an extensive enzymatic reaction can generate a wide variety of peptides, not all of them contain structural patterns that give rise to antioxidant capacity. Recent research highlights that low molecular weight peptides (<3 kDa) containing aromatic or hydrophobic amino acids (Tyr, Trp, Phe, Leu, Pro) are more efficient antioxidants (Zhang *et al.*, 2024; Zhu *et al.*, 2022).

The previous results demonstrated that microwave therapy is a promising alternative for obtaining relative soluble TCA peptides with antioxidant capacity. To visualize the potential of microwave-assisted hydrolysis for antioxidant production, treatments were performed at 600 W (T6) and 900 W (T7) without a temperature ramp. Figure 4 shows that T7 reached a higher concentration of TCA-soluble compounds (2.51 mg Tyr eq/mL) than T6 (1.45 mg Tyr eq/mL). However, this difference should not be attributed to microwave power alone, because the increase in power was accompanied by a higher final temperature (160 °C in T7 vs 130 °C in T6). Therefore, under these non-isothermal conditions, the greater release

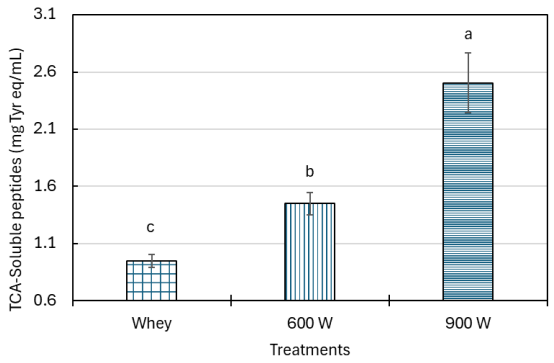


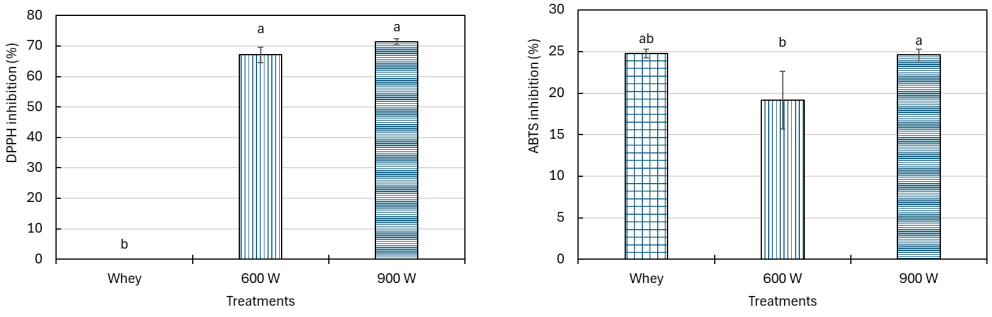
Figure 4. Comparison of relative TCA-soluble peptides in untreated whey and by microwave hydrolysis treatments without temperature ramp. Bars are the arithmetic means of 3 replicates ± the corresponding standard deviation. Means that do not share a letter are significantly different (Tukey $p < 0.05$).

of soluble material is interpreted as the result of the combined effect of higher microwave power and greater thermal severity, both of which may contribute to protein destabilization and the formation of soluble compounds. The increase in peptide content in the sample can be attributed to the rapid and homogeneous heating by the microwave method (Zhang *et al.*, 2024).

Regarding antioxidant capacity, Figure 5a shows that the control (T0) did not exhibit DPPH inhibition. In contrast, treatment T7 reached 71.5% and T6 67.1%. For ABTS inhibition (Figure 5b), the highest inhibition of the radical was observed in natural whey (24.8%), with no statistically significant difference compared to the 900 W treatment (24.6%).

The values shown suggest that antioxidant capacity depends more on the structural profile of the released compounds than on their total quantity, and that the DPPH assay may exhibit plateau effects. Ngo *et al.* (2023) demonstrated that, by their nature, whey proteins limit the exposure of aromatic and hydrophobic amino acids responsible for this reducing capacity. Yang *et al.* (2022) in an assay with milk protein at power levels below 1000 W, reported an increase in the antioxidant capacity of the hydrolysate compared to the control, due to the exposure of hydrophobic groups of the peptide. Similarly, Zhu *et al.* (2022) indicated that controlled heat treatments generate peptides with bioactivity, which contribute to the neutralization of free radicals.

According to a recent study, the polarity of the DPPH and ABTS assays differs, with DPPH being lipophilic and exhibiting a better response to hydrophobic peptides, while ABTS is hydrophilic and relies on charged or polar amino acids (Zhu *et al.*, 2022). This suggests that microwave-released peptides may preferentially bind to DPPH rather than ABTS. As Cui *et al.* (2024) reported in their study of whey hydrolysates, some pretreatments increased antioxidant capacity in DPPH but not in ABTS. Taken together, these results confirm that microwave severity increases relative TCA-soluble content (mg Tyr eq/mL), but the antioxidant response measured by DPPH/ABTS depends on the type of radical and the specific set of species generated.



(a) (b)

Figure 5. Effect of microwave power on the percentage of (a) DPPH and (b) ABTS reduction capacity. Bars are the arithmetic means of 3 replicates ± the corresponding standard deviation. Means that do not share a letter are significantly different (Tukey $p < 0.05$).

Conclusions

This study compared microwave-, ultrasound-, and enzyme-assisted hydrolysis of asadero cheese whey, evaluating the <50 kDa fraction through relative TCA-soluble peptide content and antioxidant activity measured by DPPH and ABTS inhibition assays. In the isothermal treatments, enzymatic hydrolysis with *Aspergillus niger* M4 extract (35 °C, 15 min) was the most efficient condition for maximizing the release of relative TCA-soluble peptides under mild conditions (1.28 mg Tyr-eq/mL), whereas microwave treatment at 600 W and 80 °C showed the highest combined antioxidant response within the initial treatment set

(T1–T5), considering both DPPH and ABTS assays. In the additional microwave assays performed without temperature ramp, the 900 W treatment showed the highest DPPH inhibition, indicating that treatment performance depended on the response variable and experimental set analyzed rather than on a single universal criterion. Taken together, these results position microwave-assisted hydrolysis as a rapid, chemical-free strategy for generating hydrolysates with antioxidant activity, and enzymatic treatment as a gentler alternative for maximizing peptide yield. Although the present study identified hydrolysis conditions associated with higher relative TCA-soluble content and antioxidant response, future work is required to determine whether these hydrolysates exhibit biological efficacy in plant bioassays for biostimulant activity under protected agriculture and abiotic stress conditions.

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