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Bioactivity and volatile compound evaluation in sheep milk processed by ohmic heating

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ABSTRACT

Ohmic heating may improve bioactive compounds and processing, ensuring food safety of beverages, liquid and pasty food, or liquid with solid pieces. Due to those traits, this study conducted a comparison between ohmic heating technology and conventional heating (CH), with a focus on assessing the impact of both methods on functional compounds (such as angiotensin-converting enzyme inhibition, α-amylase and α-glucosidase inhibition, and antioxidant activity) in both fresh and thawed raw sheep milk, which had been frozen for up to 3 mo. Different ohmic heating conditions were applied and compared to CH (3.33–8.33 V/cm vs. CH [73°C/15 s]). A total of 18 peptides with some functional activities were identified by MALDI-TOF mass spectrometry analysis. Ohmic heating samples presented the highest activities related to health, followed by CH and raw milk samples; antioxidant activity range was from 0.11% to 0.71%, antihypertensive activity ranged from 0.20% to 0.72%, and antidiabetic activity ranged from 0.21% to 0.79%. Of 51 volatile compounds detected, some were degraded by freezing, storing, and heating the sheep milk. This study showed for the first time that ohmic heating processing improved sheep milk bioactive peptides and preserved volatile compounds.
Key words: bioactive peptides, ohmic heating, pasteurization, sheep milk, volatile compounds

INTRODUCTION

No food matrix can be considered ideal in terms of nutrients. However, milk is one of those most balanced and nutritious foods and is considered an essential source of substances for human growth, maintenance, and well-being (Gil and Ortega, 2019). Sheep milk products are becoming more popular worldwide due to their higher nutritional value than traditional bovine dairy (Balthazar et al., 2017). These animals require less intensive farming practices compared with cows. Sheep milk has a complex balance of fatty acids and volatile compounds in amounts responsible for the unique sensory characteristics known as a sheepy bouquet (Watkins et al., 2021). Sheep milk contains considerable quantities of peptides and fatty acids exhibiting various types of bioactivity (Moatsou and Sakkas, 2019).

In addition, protein, fat, some vitamins, and minerals are present in higher amounts in sheep milk compared with cow and goat milk. Also, sheep milk (as well as goat milk) can be considered hypoallergenic, due to the size, amount, protein conformation, casein fractions, and whey proteins (Balthazar et al., 2017). Additionally, it has been documented that sheep milk casein subunits and whey proteins contain a diverse range of bioactive compounds, including antioxidants, antihypertensive agents, and antidiabetic elements, with emphasis on peptides found in the water-soluble extract (**WSE**) phase (Caroprese et al., 2019; Iram et al., 2022). Those activities can be assessed from a WSE and measured by spectrophotometric methods in different wavelengths.

Due to seasonality and low milk production per animal (Pazzola, 2019), raw sheep milk is typically stored frozen by farmers until enough is obtained for processing and manufacturing dairy products. Sheep milk can be stored for up to 6 mo without relevant changes to chemical and biochemical properties (Wendorff and Kalit, 2017).

Milk treatment is critical to avoid food contamination and foodborne diseases. More than a century ago, heating processes optimized time and temperature to establish a treatment for milk to make consumption safe. However, the conventional heating (**CH**; HTST processing, 72–75°C/15 s) process may harm the bioactivity of milk peptides. Thus, technologies have been developed and studied over time as an alternative to

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CH milk processing, such as ohmic heating (**OH**), ultrasound, high pressure, and pulsed electric field methods, among others (Alirezalu et al., 2020).

Nowadays, these emerging technologies are attracting the attention of consumers and food industries, due to health claims, minimal processing, and the potential to be less expensive than traditional methods (Gentès et al., 2022). Ohmic heating uses an external electric field applied to food products. This pasteurization technology is mainly used for processing liquid food due to its uniform and rapid heating, which also requires less energy compared with other technologies (Makroo et al., 2020). The concept of OH application relies on the material electrical conductivity that acts as a semiconductor, allowing electrical current to travel through the food matrix, dissipating volumetric heat inside the food (Pereira et al., 2018). Thus, foodborne pathogens are inactivated by the heating and electroporation phenomenon, which forces cell membrane pores to open and exchange intra- and extracellular material, leading to cell death (Makroo et al., 2020). Moreover, many studies reported that OH has no adverse effect on bioactive compounds in the food matrix, which seems to be preserved during the process because OH takes a shorter time. In contrast, CH submits food to high temperatures for long periods, which is responsible for bioactive compound degradation (Salari and Jafari, 2020).

Ohmic heating was used to process many dairy products, with a positive effect shown in respect to the bioactive peptides. For instance, Cappato et al. (2018) evaluated different operational conditions (25–80 V at 10–1,000 Hz) and verified improvement of total phenolics, 1,1-diphenyl-2-picrylhydrazyl (**DPPH**), ferric reducing antioxidant power (**FRAP**), and angiotensin-converting enzyme (**ACE**) levels on whey acerola-flavored drink after the OH process. Kuriya et al. (2020) showed that electric field intensity between 4 and 5 V/cm provided the release of bioactive peptides, with minimal damage to the rheological and sensory characteristics on blueberry-flavored dairy dessert. In addition, Ferreira et al. (2019) also verified the increase of bioactive peptides in a whey- and raspberry-flavored beverage processed by OH with antioxidant, antidiabetic, and antihypertensive activities, and volatile compounds. Therefore, OH technology could be considered advantageous for dairy processing.

To the authors' knowledge, there is no literature reporting bioactivity performed in sheep milk processed by any emerging technology, which could be interesting for further study. Thus, the objective of this study was to compare OH technology and CH for processing sheep milk, evaluating the effect of both pasteurization technologies on fresh and thawed (after being frozen for up to 3 mo) stocked raw sheep milk functional compounds, such as bioactive peptides and volatile compounds. The bioactive peptides profile was assessed by MALDI-TOF-MS, while volatile compounds were identified by GC-MS.

MATERIALS AND METHODS

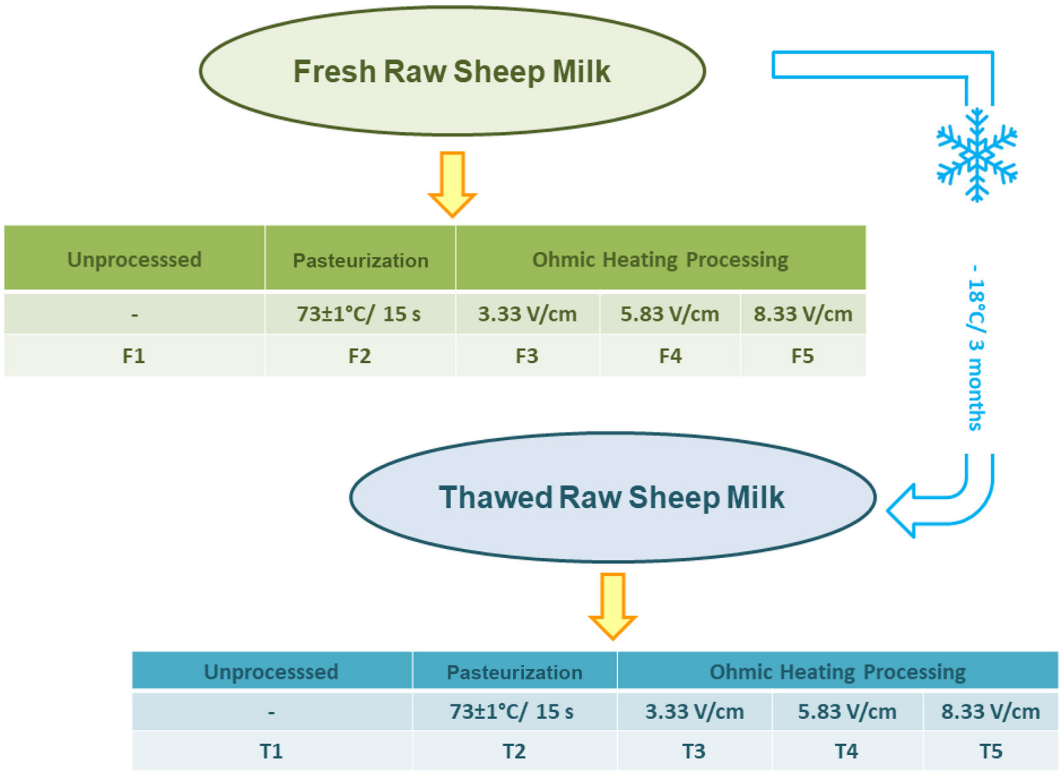
Sampling

The experiment used whole raw sheep milk of the Lacaune sheep herd located in the northeast São Paulo region, Brazil, during spring of 2019. Milk samples were obtained during routine milking procedures; therefore, Institutional Animal Care and Use Committee approval was not required.The sheep herd was composed of 73 female animals of the same age, stage of lactation, and semi-intensive feed system, where the animals received concentrate and hay and were allowed to pasture freely during the day. To ensure a good amount of milk for dairy production, the farmer milked the ewes at dawn and before twilight every day, and the bulk milk was kept refrigerated (4°C) for 3 d prior to processing. Sheep milk composition was 7.07% (wt/wt) fat, 5.2% (wt/wt) protein, 4.5% (wt/wt) lactose, and 11.1% (wt/wt) nonfat solids. Sheep milk sampling used bulk milk from 3 different moments of the same season to make triplicate batches. Each sheep milk batch was divided into 2 groups before processing: fresh (**F**) and thawed (**T**). The T group was frozen at −18°C and stored for 3 mo before being thawed and processed the same way as the F group. The F and T groups generated 5 samples each as follows: F1 and T1 (raw sheep milk); F2 and T2 (CH pasteurization of sheep milk); and F3, F4, F5, T3, T4, and T5 (OH pasteurization of sheep milk), as shown in Figure 1.

Processing Samples by CH and OH

A volume of 500 mL of raw sheep milk (4.1 mS/cm) was processed by either CH or OH. For CH, milk was placed in a stainless steel container to be heated in a water bath (99 ± 1°C); for OH, milk was placed in a polypropylene container (ohmic cell) to be directly heated. In both processes, the milk was heated to 73 ± 1°C, where it remained for 15 s, then it was immediately cooled in an ice bath (0 ± 2°C) and stored at 4 ± 2°C.

The OH cell was composed of a 1.5-L food grade polypropylene container (11 cm height, 13 cm width, 11 cm depth) and stainless steel electrodes type 316 (9 cm height, 12.5 cm width, 0.2 cm thickness). The OH system was composed of a voltage variator (TDGC2–2kVA, JNG) from 0 to 220 V, which produced experimental voltages (40, 70, and 100 V) at 60 Hz frequency



Unprocesssed	Pasteurization	Ohmic Heating Processing		
-	73±1°C/ 15 s	3.33 V/cm	5.83 V/cm	8.33 V/cm
F1	F2	F3	F4	F5

Unprocesssed	Pasteurization	Ohmic Heating Processing		
-	73±1°C/ 15 s	3.33 V/cm	5.83 V/cm	8.33 V/cm
T1	T2	T3	T4	T5

Figure 1. Flowchart of sheep milk sampling for processing by conventional heating or ohmic heating with 3 different electric field strengths.

with electric field strength of 3.33, 5.83, and 8.33 V/cm, respectively. Those voltages and electric field strengths corresponded to OH-treated samples F3 and T3, F4 and T4, and F5 and T5, respectively. For data acquisition of the control parameters, digital multimeters (ET-2042E, Minipa) were used to measure the voltage (V), temperature (°C), and electrical current (A) in the system. To preserve the freshness of processed sheep milk, samples were aliquoted and frozen (−80°C) at d 1 until being thawed at room temperature in the same day of analysis.

Analysis of Sheep Milk Peptide Bioactivity

First, the WSE were prepared for each sheep milk sample according to Ayyash et al. (2018). Samples' acidities were adjusted with 1.0 *M* HCl or 1.0 *M* NaOH to pH 4.6 and centrifuged (10,000 × *g* for 15 min at 4°C). The supernatants were filtered through a 0.45-μm syringe and stored at −20°C for further analyses.

The proteolysis analysis was performed according to Batista et al. (2017). Briefly, samples of thawed WSE were vortexed for 1 min and centrifuged at 10,000 × *g* for 5 min at room temperature. The *o*-phthaldialdehyde (**OPA**) reagent was prepared prior analysis by mixing together the following chemical substances diluted in 50 mL of Milli-Q water: 25 mL of sodium tetraborate buffer (100 m*M*, pH 9.3); 2.5 mL of SDS (20% wt/wt); 40 mg of OPA in 1 mL of methanol (wt/vol); and 100 μL of β-mercaptoethanol. Each 150 μL of sheep milk WSE was added to 1.5 mL of OPA reagent and gently mixed for 5 s until the absorbance was measured after 2 min at 340 nm using UV-spectrophotometer (Cary 60 UV-Vis Spectrophotometer). The proteolysis data were acquired by subtracting the blank (corrected absorbance) from the absorbances at 340 nm measured for each sample. Milli-Q water was used instead of a sample for the blank. The results were expressed in terms of absorbance at 340 nm.

$$\mathrm{Abs}_{340} = \mathrm{Abs}_{340sample} - \mathrm{Abs}_{340blank} \quad [1]$$

Radical scavenging rate by DPPH assay was determined according to Ayyash et al. (2018), using 800 μL of DPPH reagent (0.1 m*M* DPPH dissolved in 95% methanol), added to 200 μL of sheep milk WSE and vortexed before being left in the dark at room temperature for 30 min. The absorbance was measured at 517 nm, and the percentage of radical scavenging activity was expressed as scavenging rate (%), as Equation 2:

$$\text{Scavenging rate}\,\% = \left(1 - \frac{\text{Abs}\,sample}{\text{Abs}\,blank}\right) \times 100. \quad [2]$$

The angiotensin I-converting enzyme inhibitor (**ACEI**) activity was determined following the spectrophotometric assay (Ayyash et al., 2018), using 5 mL of sheep milk WSE dissolved in 1 mL of Tris buffer (50 m*M*, pH 8.3) and 300 m*M* NaCl. The ACE enzyme from rabbit lung (Sigma-Aldrich) and hippuryl-histidyl-leucine (**HHL**) was prepared with Tris buffer. One hundred microliters of 3.0 m*M* HHL, 100 μL of 1.25 μg/mL ACE enzyme, and 100 μL of dissolved sheep milk sample WSE were mixed and incubated at 37°C for 30 min in a water bath, then mixed and left for another 30 min in a water bath. Glacial acetic acid (200 μL) was used to stop the reaction. Then, the solution was read in a UV-visible spectroscopy detector set at 228 nm. A control reaction was mixed with 100 μL of buffer, because it was expected a liberation of maximum hippuric acid from substrate due to uninhibited ACE activity. The ACEI activity was calculated as in Equation 3 and expressed as a percentage (%):

$$\text{ACEI}\,(\%) = \left(\frac{\text{Abs}\,control - \text{Abs}\,sample}{\text{Abs}\,control}\right) \times 100. \quad [3]$$

The α-amylase and α-glucosidase inhibition activities were performed by spectrophotometric assay (Ayyash et al., 2018). The first enzyme inhibition was measured by mixing 100 μL of sheep milk samples WSE with 100 μL of α-amylase from human saliva (1.0 unit/mL, Sigma), which was pre-incubated at 37°C for 5 min in phosphate buffer (pH 6.8). The reaction was determined by addition of 200 μL of DNS reagent (1% 3,5-dinitrosalicylic acid and 12% sodium potassium tartrate in 0.4 *M* NaOH). Afterward, the solution was heated to 100°C for 15 min and diluted with 2 mL of Mili-Q water in an ice bath to stop the reaction. The absorbance was measured at 540 nm, and results were expressed in relation to sample protein/peptide content (%).

For α-glucosidase inhibition, the enzyme (1 unit/mL, Sigma) was dissolved in 100 μL of 0.1 *M* potassium phosphate buffer (pH 6.8) mixed with 50 μL of sheep milk sample WSE, incubated at 37°C for 10 min, and added with 50 μL of m*M* *p*-nitrophenyl α-D-glucopyranside (**pNPG**) for enzumatic reaction at 37°C for 30 min, which was stopped by 1 mL of 0.1 *M* Na_2CO_3 addition. The release of *p*-nitrophenyl from pNPG at 400 nm was measured to determinate the α-glucosidase activity. A solution without WSE or substrate were used as control or blank, respectively, and inhibition (%) calculated by Equation 4:

$$\text{Inhibition}\,\% = \left(1 - \frac{\text{Abs}\,sample - \text{Abs}\,blank}{\text{Abs}\,control}\right) \times 100. \quad [4]$$

Bioactive Peptide Profile Assessed by MALDI-TOF-MS

All reagents and solvents used in this analysis were analytical or MS grade and purchased from Sigma-Aldrich; water was purified using a Milli-Q system from Millipore and filtered through 0.22-μm membrane. Sheep milk samples were lyophilized and dissolved in 10 μL of acidified water (0.1% trifluoroacetic acid in water). Then 2 μL was mixed with the matrix solution (0.05 *M* α-cyano-4-hydroxy cinnamic acid in acetonitrile/ethanol [1:1 vol/vol]). Subsequently, 0.5 μL of this mixture was placed on a MALDI target plate (MTP 384, Bruker Daltonics) and dried at room temperature.

Bioactive peptide profiles were obtained by high-resolution MALDI-TOF-MS. The equipment used was an Autoflex maX MALDI mass spectrometer (Bruker Daltonics) equipped with a 355 nmNd:YAG laser. Mass spectra were acquired in positive reflection mode using an accelerating voltage of 19 kV and a laser frequency of 1 kHz. The ion detection range was m/z 0.7 to 3.5 kDa. External calibration was performed using a standard peptide mixture. Data were acquired using Flex Control software (flexcontrl 3.4, Bruker Daltonics), and spectra were processed using Flex Analysis software (version 3.4, Bruker Daltonics). It was applied the Swissprot (BLASTp tool) for peptides sequences identification and the bioactive peptides were identified through homology according to the milk bioactive peptide database (http://mbpdb.nws.oregonstate.edu/; Nielsen et al., 2017).

Volatile Compounds of Sheep Milk

The volatile compounds were identified by GC-MS (GC-MS gas chromatograph, Varian 3800, Sunnyvale, CA) with direct interface with the Varian 2000 (Varian Spa Mass Spectrometer). Solid-phase microextractions were done with divinylbenzene, carboxen, and polydimethylsiloxane fibers of 50/30 μm thickness and 20-mL headspace flasks in an automated CTC PAL sampler (Supelco). The analysis conditions were carried out according to the method described previously by Balthazar et al. (2021), with CP-Wax 52 CB column (60 m, 0.25 mm i.d., film thickness of 0.25 mm). The linear retention index of the compounds was calculated according to the van Den Dool and Kratz (1963) equation and compared with the linear retention index standards of the C8–C40 alkanes (Supelco).

Table 1. Mean ± SD of proteolysis (A_{340}), α-amylase and α-glucosidase inhibition (%), angiotensin-converting enzyme inhibition (ACEI, %), and antioxidant activity (1,1-diphenyl-2-picrylhydrazyl, DPPH; %) in fresh (F) and thawed (T) raw, conventional heating, and ohmic heating pasteurized sheep milk at refrigerated storage (4°C ± 1), and the variation related to the fresh raw sheep milk (Δ_{F1}) after freeze or processing

Sample[1]	Proteolysis	Δ_{F1}	α-Amylase	Δ_{F1}	α-Glucosidase	Δ_{F1}	ACEI	Δ_{F1}	DPPH	Δ_{F1}
F1	0.44 ± 0.02^{e}	1.00	0.42 ± 0.02^{e}	1.00	0.38 ± 0.02^{e}	1.00	0.32 ± 0.02^{g}	1.00	0.24 ± 0.02^{g}	1.00
F2	0.56 ± 0.02^{d}	1.28	0.57 ± 0.04^{c}	1.38	0.47 ± 0.02^{d}	1.23	0.47 ± 0.02^{e}	1.46	0.45 ± 0.02^{e}	1.91
F3	0.83 ± 0.02^{a}	1.90	0.73 ± 0.02^{b}	1.74	0.69 ± 0.02^{b}	1.81	0.66 ± 0.03^{c}	2.06	0.65 ± 0.02^{c}	2.75
F4	0.80 ± 0.03^{ab}	1.83	0.79 ± 0.03^{a}	1.88	0.70 ± 0.03^{b}	1.85	0.69 ± 0.02^{b}	2.16	0.71 ± 0.02^{a}	3.01
F5	0.79 ± 0.02^{b}	1.81	0.71 ± 0.02^{b}	1.71	0.76 ± 0.02^{a}	2.00	0.53 ± 0.02^{d}	1.65	0.68 ± 0.02^{b}	2.87
T1	0.34 ± 0.02^{f}	0.78	0.35 ± 0.03^{f}	0.84	0.21 ± 0.03^{g}	0.54	0.20 ± 0.03^{h}	0.63	0.11 ± 0.03^{i}	0.45
T2	0.43 ± 0.03^{e}	0.98	0.41 ± 0.03^{e}	0.99	0.34 ± 0.02^{f}	0.89	0.35 ± 0.03^{f}	1.11	0.24 ± 0.02^{g}	1.00
T3	0.74 ± 0.07^{c}	1.70	0.59 ± 0.04^{c}	1.40	0.55 ± 0.03^{c}	1.44	0.72 ± 0.03^{a}	2.27	0.41 ± 0.02^{f}	1.73
T4	0.73 ± 0.07^{c}	1.69	0.51 ± 0.03^{d}	1.22	0.54 ± 0.03^{c}	1.43	0.71 ± 0.03^{ab}	2.22	0.52 ± 0.02^{d}	2.23
T5	0.71 ± 0.04^{c}	1.63	0.50 ± 0.02^{d}	1.20	0.44 ± 0.05^{d}	1.15	0.71 ± 0.03^{ab}	2.22	0.45 ± 0.02^{e}	1.89

[a–i]Different letters in the same column indicate a significant difference ($P < 0.05$).

[1]F1 and T1 = raw sheep milk; F2 and T2 = conventional heating pasteurized (73°C/15 s) sheep milk; F3 and T3 = ohmic heating pasteurized sheep milk (3.33 V/cm); F4 and T4 = ohmic heating pasteurized sheep milk (5.83 V/cm); F5 and T5 = ohmic heating pasteurized sheep milk (8.33 V/cm).

Statistical Analysis

The study was performed in a completely randomized design with 3 experimental replications, with significant variation observed between experiment replicates ($P > 0.05$, data not shown). The analyses of each experimental replication were carried out in triplicate. The data were evaluated in XLSTAT version 2022.2 (Addinsoft), using ANOVA and Fisher's test ($P \leq 0.05$) as mean ± standard deviation. A principal component analysis (**PCA**) bidimensional map was used considering a matrix dataset with 10 rows and 5 columns (samples × bioactive compounds and proteolysis values) using the correlation matrix. Confidence ellipses using bootstrapping method (n = 500 resamplings) were also generated to check the similarity of the parameter's values of the treatments; confidence ellipses allow a complete reading of the PCA bidimensional map because they give a graphical approach to the significance of the difference between the products and their parameters. The data were previously autoscaled to assign a similar weight for the variables and eliminate possible effects of the numerical values. This approach has been typically used in problems in the dairy industry in recent studies (Pathania et al., 2021).

RESULTS AND DISCUSSION

Bioactivity of Sheep Milk

Table 1 shows the sheep milk bioactivity represented by proteolysis (OPA assay), antidiabetic (α-amylase and α-glucosidase inhibitions assays), antihypertensive (ACEI assay), and antioxidant (DPPH radical scavenging activity assay) activities in fresh and thawed, raw and processed samples. The variation of bioactivity is also reported in relation to fresh raw sheep milk (ΔF1) as a control to better visualize the impact of those above-mentioned activities.

It could be verified (Table 1) that milk processing significantly improved bioactivity, with the OH process always superior to CH ($P < 0.05$). The freezing storage up to 3 mo or thawing procedure had a negative effect on bioactivity. The bioactivities analyzed in sheep milk were demonstrated to be higher in fresh than in thawed samples ($P < 0.05$), with the exception of ACEI. The hypertensive values of OH thawed samples were more than twice the value of F1 ($P < 0.05$). Thus, whenever possible, it is better to process fresh sheep milk to achieve greater bioactive improvement instead of freezing it first.

It is difficult to state which electric field strength best improved sheep milk bioactivity, because no electric field strength showed better performance for all bioactivities evaluated. For instance, electric field strength of samples F3 and F4 almost duplicated the proteolysis activity (1.83–1.90 > F1, $P < 0.05$), F4 and F5 also almost duplicated the antidiabetic activity (α-amylase and α-glucosidase inhibition 1.71–2.00 > F1, $P < 0.05$), thawed ohmic heated samples more than duplicated the antihypertensive activity (2.22–2.27 > F1, $P < 0.05$), and F4 triplicated the initial amount of antioxidant activity (3.01 > F1, $P < 0.05$; Table 1).

The bioactivity improvement in sheep milk after OH processing could be explained by the electricity accelerating the proteolysis breakdown of milk protein, which generates various peptides with biological activities (antimicrobial, antioxidant, antihypertensive, antithrombotic, and immunomodulatory; Gan et al., 2019). According to Greenwood and Honan (2019), the high degree of proteolysis is related to the development of bioactive peptides in milk, which is interesting from

a functional point of view. However, a high proteolysis level in milk could not be desired for some dairy product production, due to its interference in technological properties such as cheeses texture (Gumus and Hayaloglu, 2019).

Contrarily, Kondyli et al. (2022) studied the effect of mushroom fortification polysaccharide β-glucan on the quality of ovine soft spreadable cheese and verified that proteolysis levels didn't affect the sensory properties of cheeses manufactured. Thus, it could be concluded that the release of peptides because of proteolysis could improve the healthy functional parameters in milk and dairy products.

Proteolysis is the hydrolysis of proteins into peptides and AA, and the proteolytic behavior in food could be correlated with bioactive peptides presented or released during processing or storage. Thus, after conventional or OH processing on the first cold storage day of fresh and thawed sheep milk, proteolysis and bioactivities were evaluated in sheep milk. In addition, DPPH free radical is commonly used to estimate the antioxidant activity of certain compounds by using scavenged electron donating (Baliyan et al., 2022).

Peptides exhibiting ACEI activity are often measured as antihypertensive agents and considered a therapeutic approach for hypertension treatment (Amorim et al., 2022). The ACEI peptides are released from casein or whey protein by the digestive enzymes, microbial and plant enzymes, or proteolytic activities of starter probiotic bacteria during milk fermentation (Balthazar et al., 2018). In addition, milk could present a low level of ACEI due to intrinsic proteolytic enzymes of other mechanisms that release those peptides, as shown in sheep milk processed by OH.

Moreover, the inhibition of α-amylase and α-glucosidase activity can be considered an effective approach to controlling diabetes via diminishing carbohydrate hydrolysis, attributed to bioactive peptides factions lower than 3 kDa (Ayyash et al., 2018).

Balthazar et al. (2022) discussed the advantages and disadvantages of electric field strengths (3.33, 5.83, or 8.33 V/cm) related to milk microbiological quality and energy consumption during sheep milk processing. In addition, the lower activity of biopeptides in CH could be related to protein denaturation at high temperatures (Alirezalu et al., 2020), due to the high heating rate of CH pasteurization (F2 and T2 = 8.28°C/min; F3 and T3 = 0.58°C/min; F4 and T4 = 3.48°C/min; F5 and T5 = 6.75°C/min; Balthazar et al., 2022). The mechanism responsible for bioactive compound improvement on sheep milk OH processing might be related to the electroporation phenomenon caused by electric field strength. When applied, the electric field opens cell membrane pores, releasing intracellular enzymes responsible for protein cleavages, such as casein fractions and whey protein (Salari and Jafari 2020).

A PCA bidimensional map was used to understand and illustrate the relationship between bioactivity in sheep milk and samples from this study (Figure 2), explaining 90.18% of data in the F1 dimension and 8.26% F2 dimension. Sheep milk bioactivity and OH samples were located closely in the first and fourth quadrants of PCA, corroborating with data results in Table 1, and the statement that emerging technology was found to improve functional compounds in food compared with traditional processing technologies (Galanakis, 2021).

The mechanism of bioactive compounds released in the food matrix during OH processing is driven by the hydrolysis increase and changes in protein denaturation, inducing small peptide formation (Ferreira et al., 2019; Kuriya et al., 2020). Lower molecular weight peptides between 756 and 1,140 Da exhibit antihypertensive activity, and those between 1,392 and 1,881 Da are linked to antioxidant activity, commonly obtained from β-CN (Rao et al., 2020). The α-amylase and α-glucosidase indicated an antidiabetic activity, which could be attributed to the presence of bioactive peptides binding components with affinity to those enzymes site (Ferreira et al., 2019).

Functional peptides appear in low concentration in milk, but these could be raised in dairy products by probiotic bacteria fermentation (Virtanen et al., 2007; Li et al., 2015; Batista et al., 2017; Ayyash et al., 2018; Balthazar et al., 2019, 2021; Ferreira et al., 2019). Some studies have shown bioactive compounds enhanced after OH processing (Cappato et al., 2018; Ferreira et al., 2019; Kuriya et al., 2020) as previously cited. However, none of those are reported in nonbovine milk products or milk stocked frozen. This specific habit is common among small-ruminant farmers to accumulate enough milk for processing and producing dairy products (Balthazar et al., 2017; Wendorff and Kalit, 2017). Therefore, observations that OH processing improves the evaluated peptides bioactivities in sheep milk expands the horizons of this technology application to enhance the healthy functionality of dairy products.

Peptide Profile of Sheep Milk Assessed by MALDI-TOF-MS

Analysis by MALDI-TOF-MS has been essential in identifying and correlating peptides concerning their functionality in the food matrix. The sheep milk peptides profile was assessed by MALDI-TOF-MS analysis, showing the mass spectra of bioactive peptides (Figure 3). A total of 18 peptides with some functional activities were identified in fresh or thawed sheep milk samples, as described in Table 1. Most signals were detected in

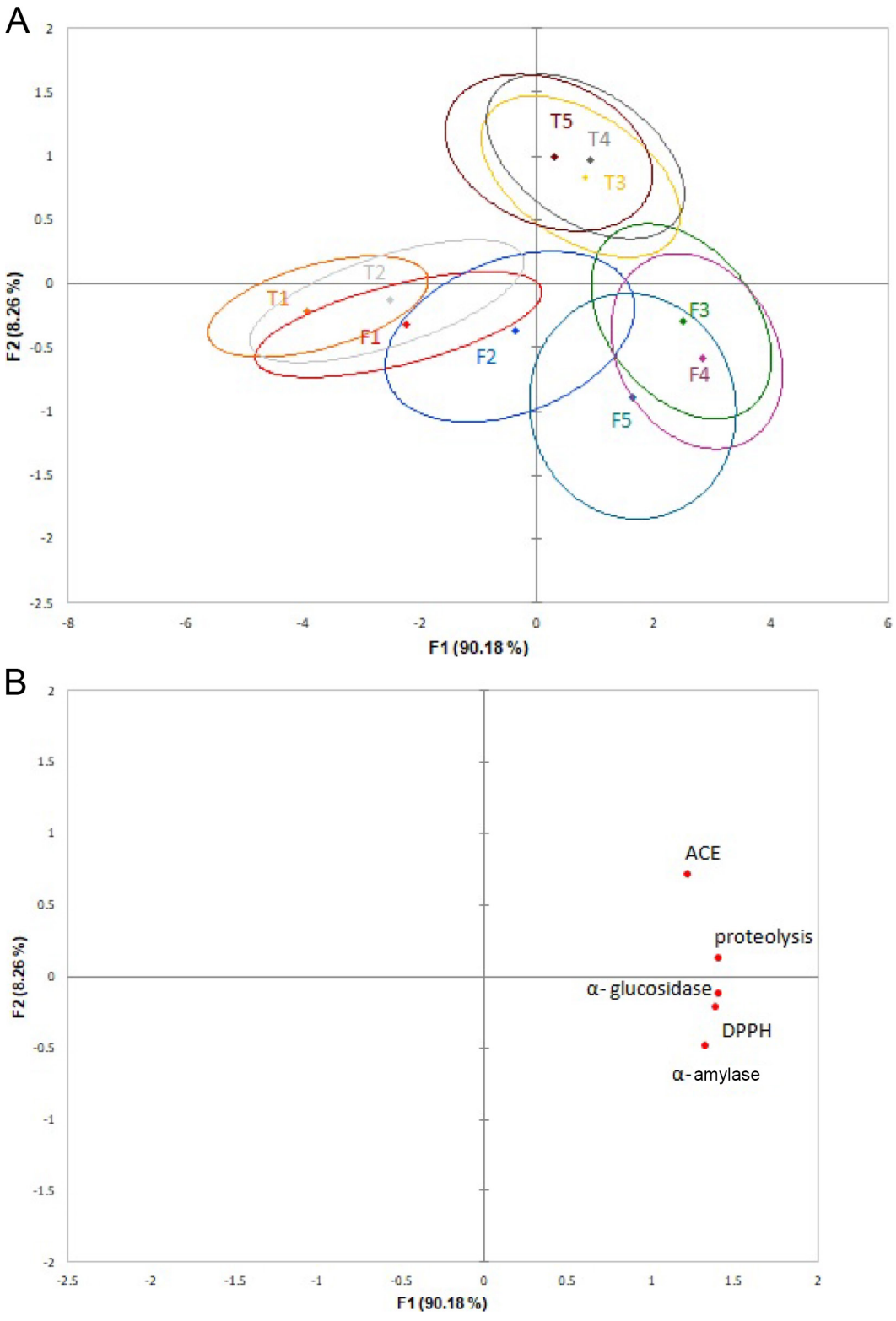


Figure 2. Principal component analysis (PCA) of bioactive compounds in fresh (F) and thawed (T) sheep milk pasteurized by conventional heating (CH) and ohmic heating (OH) applying different electric field strength. Shown are 2-dimensional distribution of samples (A) and enzyme assays (B) of antidiabetic (α-amylase and α-glucosidase), antihypertensive (ACE), and antioxidant (DPPH) activities. F1 and T1 = raw sheep milk; F2 and T2 = CH pasteurized sheep milk; F3 and T3 = OH pasteurized sheep milk (3.33 V/cm); F4 and T4 = OH pasteurized sheep milk (5.83 V/cm); F5 and T5 = OH pasteurized sheep milk (8.33 V/cm). DPPH = 1,1-diphenyl-2-picrylhydrazyl; ACE = angiotensin-converting enzyme.

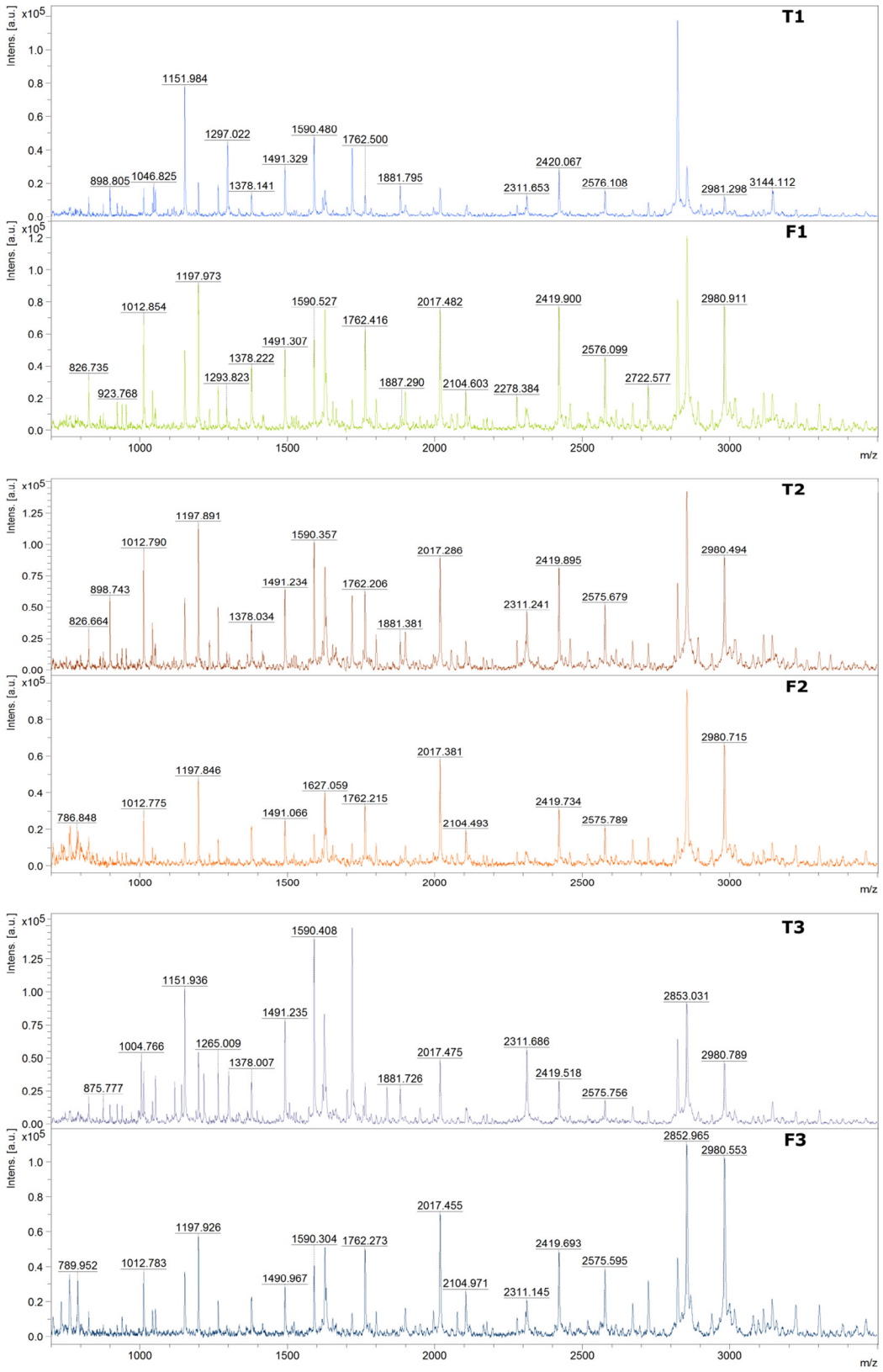


Figure 3. Schematic chart of mass spectra of bioactive peptides obtained by MALDI-TOF-MS in sheep milk. a.u. = arbitrary units related to signal intensity. F1 and T1 = raw sheep milk; F2 and T2 = CH pasteurized sheep milk; F3 and T3 = OH pasteurized sheep milk (3.33 V/cm); F4 and T4 = OH pasteurized sheep milk (5.83 V/cm); F5 and T5 = OH pasteurized sheep milk (8.33 V/cm).

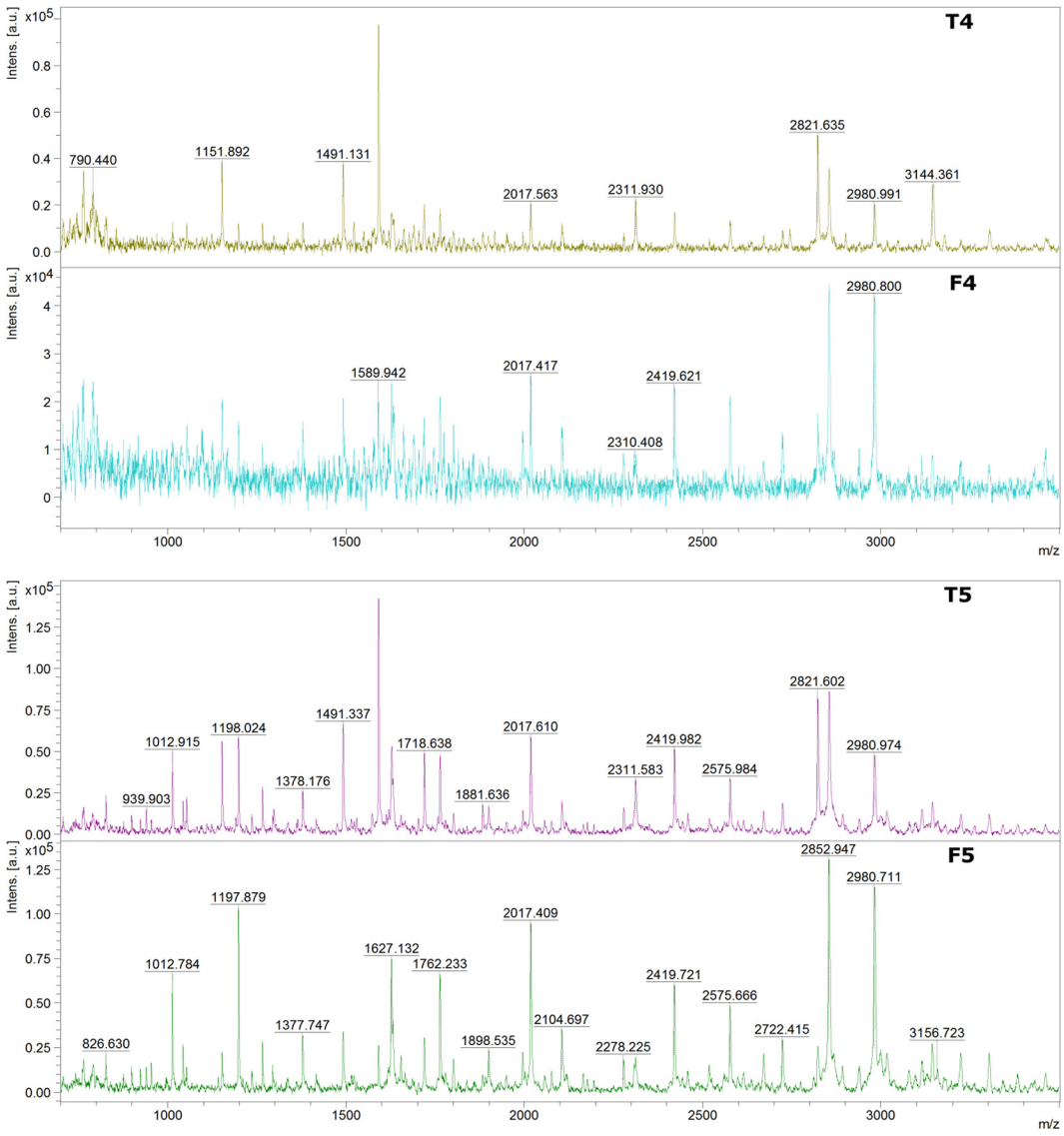


Figure 3 (Continued). Schematic chart of mass spectra of bioactive peptides obtained by MALDI-TOF-MS in sheep milk. a.u. = arbitrary units related to signal intensity. F1 and T1 = raw sheep milk; F2 and T2 = CH pasteurized sheep milk; F3 and T3 = OH pasteurized sheep milk (3.33 V/cm); F4 and T4 = OH pasteurized sheep milk (5.83 V/cm); F5 and T5 = OH pasteurized sheep milk (8.33 V/cm).

a range of 800 to 2,000 Da, corresponding to approximately 10 to 20 AA peptide lengths. These peptides resulted from casein protein fraction (α-CN, β-CN, and κ-CN) and whey protein (β-LG). Specifically, α-CN originated 5 peptides with antibacterial (RPKHPIK: Rizzello et al., 2005) and antihypertensive (NENLLRFF: Yoshikawa et al., 1994; IGSENSEKTTMP: Hayes et al., 2007; RPKHPIKHQ: Saito et al., 2000; FFVAPFPEVFGK: Yamada et al., 2015) activities. From β-CN it was derived 7 peptides with anti-anxiety, anticancer, antioxidant, and opioid activity (YPFPGPI: Wang et al., 2012; Zoghbi et al., 2006), antibacterial and anticoagulant (YQEPVLGPVRGPFPIIV: Rojas-Ronquillo et al., 2012), antihypertensive (HQPHQPLPPT and EPVLGPVRGPFP: Adams et al., 2020; QEPVLGPVRGPFPIIV: Lu et al., 2016; QSLVYP: Chang and Alli, 2012) and immunomodulatory (LYQEPVLGPVRGPFPIIV: Coste et al., 1992) activities. From κ-CN emerged 4 peptides with antihypertensive (IESPPEIN: Coste et al., 1992; VLSRYP: Adams et al., 2020), antioxidant (INNQFLPYPYYAKPA: Tonolo et al., 2020), and opioid (SRYPSY: Yoshikawa et al., 1994) activities. Finally, β-LG released 2 peptides with antibacterial (AASDISLLDAQSAPLR: Pellegrini et al., 2001) and antilipolytic (QKVAGTW: Lacroix et al., 2016) activities.

Table 2. Biopeptides identified in sheep milk samples, presenting the protein's origin, sequences, mass-to-charge ratio (m/z), and biological activity

Sample[1]	Protein origin	Sequence	m/z	Biological activity[2]	Reference
F1, T1, T2, T3, F5	α-CN	RPKHPIK	875	Antibacterial	Rizzello et al. (2005)
F1, T1, T2, F3, T3, T4, T5		NENLLRFF	1,052	ACE inhibition	Yoshikawa et al. (1994)
F1, T1		IGSENSEKTTMP	1,293	ACE inhibition	Hayes et al. (2007)
T3		RPKHPIKHQ	1,140	ACE inhibition	Saito et al. (2000)
F2		FFVAPFPEVFGK	1,384	ACE inhibition	Yamada et al. (2015)
All	β-CN	HQPHQPLPPT	1,151	ACE inhibition	Adams et al. (2020)
All		EPVLGPVRGPFP	1,264	ACE inhibition	Adams et al. (2020)
F2, F3, T3, F4, T4, F5		YPFPGPI	790	Anti-anxiety, anticancer, antioxidant, opioid	Wang et al. (2012); Zoghbi et al. (2006)
F1, T1, T2, T3, T4, F5		QEPVLGPVRGPFPIIV	1,718	ACE inhibition	Lu et al. (2016)
T2		QSLVYP	706	ACE inhibition	Chang and Alli (2012)
T2, F3		LYQEPVLGPVRGPFPIIV	1,994	Immunomodulatory	Coste et al. (1992)
T1, T2, T3, T5		YQEPVLGPVRGPFPIIV	1,881	Antibacterial, anticoagulant, immunomodulatory	Rojas-Ronquillo et al. (2012)
T1, T2, T3, T5	κ-CN	IESPPEIN	898	ACE inhibition	Coste et al. (1992)
F2, F4		VLSRYP	734	ACE inhibition	Adams et al. (2020)
F5		INNQFLPYPYYAKPA	1,799	Antioxidant	Tonolo et al. (2020)
T2		SRYPSY	772	Opioid	Yoshikawa et al. (1994)
F1, F5, T5	β-LG	AASDISLLDAQSAPLR	1,627	Antibacterial	Pellegrini et al. (2001)
T2, F3		QKVAGTW	789	Antidiabetic	Lacroix et al. (2016)

[1]Samples of fresh (F) and thawed (T) sheep milk were numbered 1 to 5. F1 and T1 = raw sheep milk; F2 and T2 = conventional heating pasteurized (73°C/15 s) sheep milk; F3 and T3 = ohmic heating pasteurized sheep milk (3.33 V/cm); F4 and T4 = ohmic heating pasteurized sheep milk (5.83 V/cm); F5 and T5 = ohmic heating pasteurized sheep milk (8.33 V/cm).
[2]ACE = angiotensin-converting enzyme.

All sheep milk samples presented the peptides HQPHQPLPPT and EPVLGPVRGPFP. Those peptides present antihypertensive activity derived from β-CN (Adams et al., 2020). This fact means that some sheep milk peptides may express antihypertensive activity, whether fresh, thawed, raw, or pasteurized. Nevertheless, some peptides were generated in some thawed sheep milk samples (T1 to T5), indicating that the freezing process may be influencing proteolysis and protein rehydration releasing peptides (Pax et al., 2023). The peptides released were RPKHPIKHQ (α-CN), YQEPVLGPVRGPFPIIV (β-CN), and IESPPEIN (κ-CN), or combined with further CH as QSLVYP (β-CN) and SRYPSY (κ-CN; Table 2).

Volatile Compounds of Sheep Milk

A total of 51 volatile compounds were identified in sheep milk samples, precisely 15 acids, 13 esters, 8 hydrocarbons, 3 alcohols, 4 aldehydes, 2 ketones, and 6 compounds from other nonspecific chemical groups (Table 3). Acids were the prevalent chemical group presented in sheep milk samples; however, there were significant differences in the acid diversity between samples as follows: F1 had 15 acids; T1 and F3 had 10; F4 and F5 had 9; T3, T4, and T5 had 8; F2 had 7; and T2 had 5 ($P < 0.05$). Ketones were verified only in raw samples (F1and T1). The F1 group presented a total of 39 volatile compounds, whereas T1 had 30, F2 and T2 had between 11 and 13, and OH samples had between 14 and 18, with a significant difference in the compounds detected between these 3 groups ($P < 0.05$). Those results indicated that freezing and heating were responsible for degrading 10 and 20 volatile compounds initially present in fresh raw sheep milk (F1). Watkins et al. (2021) stated that heat treatment has little effect on sheep milk flavor perception, probably because octanoic acid, responsible for sheepy flavor (Watkins et al., 2021), is not influenced by freezing/thawing or heating, being detected is all samples of this study.

In addition, it is plausible to indicate some volatile compounds as cryo and thermolabile due to these being presented only in sample F1, being 3 acids (undecanoic, dodecanoic, tetradecanoic acids), 2 esters (ethyl 9-decanoate and tetrahydro-6-propyl-2H-pyran-2-one) and 1 ketone (2-nonanone), 1 hydrocarbon (2,4,6-trimethyl-octane) and 5-hydroxy-2,4-di-t-butyl phenyl ester pentanoic acid. Inasmuch, 6 esters (ethyl ester butanoic acid, ethyl ester hexanoic acid, ethyl ester octanoic acid, ethyl ester decanoic acid, 3-methyl butyl ester pentadecanoic acid, and 2-methyl-tetradecanoic acid) could be considered only thermolabile as well the following compounds: 9-decenoic acid, ethanol, 2,4-dimethyl-benzaldehyde, 2-octanone methyl ester,

Table 3. Volatile compounds identified in fresh (F) and thawed (T) sheep milk at first day of refrigerated storage (4°C ± 1)

			Sample[1]									
Compound	RT (min)	LRI	F1	T1	F2	T2	F3	T3	F4	T4	F5	T5
Acid												
Acetic acid	16	1,426	X		X		X					
Butanoic acid	20	1,591	X	X	X	X	X	X	X	X	X	X
3-Methyl-butanoic acid	22	1,641	X	X	X	X	X	X	X	X	X	X
Pentanoic acid	23	1,703	X	X			X	X	X	X	X	X
Hexanoic acid	25	1,804	X	X	X	X	X	X	X	X	X	X
4-Methylhexanoic acid	27	1,885	X	X							X	X
Heptanoic acid	28	1908	X	X			X	X	X	X	X	X
Octanoic acid	30	2025	X	X	X	X	X	X	X	X	X	X
Nonanoic acid	32	2,116	X	X	X	X	X	X	X	X	X	X
n-Decanoic acid	34	2,224	X		X		X		X		X	
9-Decenoic acid	35	2,283	X	X								
Undecanoic acid	36	2,339	X									
Benzoic acid	36	2,382	X	X			X	X	X	X		
Dodecanoic acid	37	2,438	X									
Tetradecanoic acid	41	2,614	X									
n-Hexadecanoic acid	47	2,861										
Alcohol												
Ethanol	6	1,071	X	X								
4-Methyl-1-hexanol	18	1,491	X	X			X	X				
3,4-Dimethyl-1-pentanol	20	1,575							X	X	X	X
Phenylethyl alcohol	27	1,889										
Aldehyde												
2-Methyl-pentanal	13	1,294							X	X	X	X
Benzaldehyde	19	1,528	X	X	X	X	X	X	X	X	X	X
2,4-Dimethyl-benzaldehyde	25	1,807	X	X								
Isophthalaldehyde	26	1,843			X	X	X	X	X	X	X	X
Esther												
Ethyl ester butanoic acid	7	1,090	X	X								
Ethyl ester hexanoic acid	12	1,267	X	X								
Butyl ester butanoic acid	13	1,316					X	X				
2-Methylpropyl ester butanoic acid	13	1,318							X	X	X	X
Ethyl ester octanoic acid	17	1,447	X	X								
Isopentyl hexanoate	17	1,475	X	X					X	X	X	X
Ethyl ester nonanoic acid	19	1,533										
Isobutyl ester decanoic acid	24	1,738										
Ethyl ester decanoic acid	21	1,631	X	X								
3-methylbutyl ester octanoic acid	22	1,654										
Ethyl 9-decenoate	22	1,679	X									
Ethyl ester undecanoic acid	23	1,724										
Isobutyl ester decanoic acid	24	1,738	X	X					X	X		
Ethyl ester dodecanoic acid	26	1,819	X	X					X	X		
3-Methylbutyl ester pentadecanoic acid	26	1,837	X	X								
Methyl ester 2-methyl-etradecanoic acid	30	2,018	X	X								
Tetrahydro-6-propyl-2H-pyran-2-one	33	2,177	X									
Ketone												
4-Octanone	22	1,671										
2-Octanone	29	1,995	X	X								
2-Nonanone	29	1,995	X									
Hydrocarbon												
2,2,3,4-Tetramethyl pentane	6	1,083					X	X				
Propyl cyclopropane	9	1,151					X	X				
3,3-Dimethyl-hexane	21	1,605	X	X								
2,7-Dimethyl-octane	22	1,680										
2,4,6-Trimethyl-octane	23	1,690	X									
3,4-Dimethyl-heptane	23	1,718			X	X						
2,3,4-Trimethyl-pentane	24	1,750									X	X
3,7,11,15-Tetramethyl-2-hexadecene	26	1,854	X	X								
8-Methyl-1-decene	26	1,854	X	X					X	X		
3,3-Dimethyl-pentane	27	1,895										
Other												
N,N-Dinonyl-2-(2-thiophenyl)-ethylamine	6	1,077			X	X						
1-Nitro-propane	8	1,099			X	X						
Butyl isohexyl ester sulfurous acid	17	1,465										

Continued

Table 3 (Continued). Volatile compounds identified in fresh (F) and thawed (T) sheep milk at first day of refrigerated storage (4°C ± 1)

Compound	RT (min)	LRI	Sample[1]									
			F1	T1	F2	T2	F3	T3	F4	T4	F5	T5
Fluorotrinitro-methane	19	1,523										
3,3-Dimethyl-1,2-epoxybutane	23	1,711										
2-Methyl-anhydride pentanoic acid	29	1,960		X								
Chlorodifluoroacetate 2,2-dimethyl-3-pentanol	25	1,785										
5-Hydroxy-, 2,4-di-t-butylphenyl ester pentanoic acid	34	2,255	X									
10-[(Tetrahydro-2H-pyran-2-yl)oxy]-1-decanol	36	2,371	X	X								
2-Chloroethyl benzoate	36	2,383	X	X								

[1]Samples of fresh (F) and thawed (T) sheep milk were numbered 1 to 5. F1 and T1 = raw sheep milk; F2 and T2 = conventional heating pasteurized (73°C/15 s) sheep milk; F3 and T3 = ohmic heating pasteurized sheep milk (3.33 V/cm); F4 and T4 = ohmic heating pasteurized sheep milk (5.83 V/cm); F5 and T5 = ohmic heating pasteurized sheep milk (8.33 V/cm). An X marks the presence of a chemical compound in sheep milk samples. RT = retention time in minutes. LRI = linear retention index.

3,3-dimethyl-hexane and 3,7,11,15-tetramethyl-2-hexadecene, 10-[(tetrahydro-2H-pyran-2-yl)oxy]-1-decanol and 2-chloroethyl benzoate, due to all those compounds were also identified in sample T1.

The freezing–thawing and heating processing were also responsible for generating 2-methyl-anhydride pentanoic acid and 11 volatile specimens, respectively. The heating process influences volatile compound generation, most of which are formed at temperatures from 60°C. The mechanism for volatile formation could be thermo-oxidation (Xiao et al., 2020) or cholesterol thermal degradation as some aldehydes (Raczyk et al., 2017). There were identified an aldehyde (isophthalaldehyde) generated in all pasteurized samples. In addition, an ester, 3 hydrocarbons, and 2 other compounds were also formed in pasteurized samples as shown in Table 3.

3,4-Dimethyl-heptane, N, N-dinonyl-2-(2-thiophenyl)-ethylamine, and 1-nitro-propane were identified only in CH samples, as well could be explained by high heating rate (8.3°C/min, Balthazar et al., 2022) applied to samples F2 and T2. Although 9 compounds were present in OH samples, suggesting formation by different electric field strengths applied. Due to that, it was possible to see volatile compounds identified in all OH samples and others present specifically in samples with the same electric field strength, for example, butyl ester butanoic acid in F3 and T3 and 2,3,4-trimethyl-pentane in F5 and T5, respectively samples which were applied the lowest (3.33 V/cm) and the highest (8.33 V/cm) electric field strength. The mechanism of volatile compounds generated by OH is not established (Hradecky et al., 2017).

CONCLUSIONS

This study is the first to compare the impact of OH processing on fresh and thawed sheep milk, focusing on identifying bioactive peptides through proteomic analysis and profiling volatile compounds. Peptides from casein and β-LG were linked to a wide range of health-related activities, and this emerging technology significantly increased antioxidant, antihypertensive, and antidiabetic peptide activities due to enhanced proteolysis when compared to conventional heating and raw samples. However, the ideal OH parameter remains undetermined, because all OH samples showed substantial bioactivity improvement. As well, profiling volatile compounds was critical for flavor and quality. Ohmic heating appears promising for sheep milk pasteurization, retaining bioactive peptides and volatile compounds, which could enhance the sheep milk industry and its dairy products, offering potential health benefits.

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