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Effects of preservative, storage time, and temperature of analysis on detailed milk protein composition determined by reversed-phase high-performance liquid chromatography

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ABSTRACT

Milk preservative and freezing are used as strategies to prevent microbial growth and milk degradation, especially when immediate analytical processing is not feasible. The effects of the addition of preservative and freezing procedures have been investigated mainly in relation to milk gross chemical composition predicted through mid-infrared spectroscopy. This study aimed to determine whether different preservatives (i.e., no preservative, hydrogen peroxide, Bronopol, and Azidiol), freezing times (i.e., 0, 7, and 30 d), and temperatures of analysis (i.e., 5 and 21°C) influence the composition of milk protein fractions determined through reversed-phase HPLC. Bulk milk samples for the analysis of protein profile were collected from 5 commercial dairy farms. Data were analyzed with a linear mixed model, which included type of preservative, time of storage, temperature of analysis, and the interaction between type of preservative and time of storage as fixed effects, with the farm and the residual as random effects. Samples with no preservative had the greatest amount of all protein fractions, whereas Bronopol-preserved milk had the lowest amount. Increasing storage time under freezing conditions had a nonlinear detrimental effect on milk protein fractions. The temperature of analysis significantly contributed to the variation of κ-casein, β-casein, α_{S1}-casein, β-lactoglobulin, and α-lactalbumin fractions. The z-scores were calculated to evaluate the similarity between detailed protein profile of fresh milk without preservative analyzed at 5°C and detailed protein profile of milk treated according to the tested conditions. Overall results suggested a good agreement between different analytical conditions. Still, short storage time under freezing conditions is recommended to avoid degradation of milk protein fractions and consequent analytical underestimation.

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INTRODUCTION

The main purpose of the addition of preservative to milk is to ensure the maintenance of the original composition from the time of milking to the time of analysis (Zajác et al., 2016). Considering that milk contains many essential nutrients and around 85% water, it becomes an ideal medium for rapid proliferation of microorganisms. Indeed, in both long stored refrigerated samples and uncooled raw milk samples, growth of bacteria and surface mold can be a problem. For this reason, preservative mixtures are commonly used to prevent sample spoilage (Saha et al., 2002). In addition to the ability to minimize bacterial proliferation, Kroger (1985) detailed the following requirements that milk preservatives should meet: broad-spectrum activity; effectiveness at low concentrations; high water solubility; stability under most storage conditions; color for safety purposes; compatibility with high- or low-fat milks; reasonably long shelf-life activity; nonallergenic, nontoxic, and nonenvironmentally hazardous; cost-effective and readily available; easily dispensed. To satisfy all these characteristics, various preservatives have been tested in the past. Historically, mercuric chloride ($HgCl_2$), potassium dichromate ($K_2Cr_2O_7$), and formalin (CH_2O) have been commonly used as milk preservatives. However, because of environmental and safety implications, their use has been discontinued and interest in alternative preservatives increased.

Newer preservatives such as Azidiol (a combination of sodium azide and chloramphenicol) have been proposed, and they are currently used in some countries (Singh and Gandhi, 2015), but with considerable environmental and safety issues. Sodium azide is indeed a toxic compound and does not degrade in the environment; moreover, the possibility of spontaneous explosion caused by the reaction between sodium azide and metals in waste system pipes, led many laboratories to abandon its use (Barbano et al., 2010). Cur-

rently, Bronopol (2-bromo-2-nitro-1,3-propanediol), a formaldehyde-releasing preservative, is the most extensively used alternative, due to its noncorrosive features (Upadhyay et al., 2014). In addition, hydrogen peroxide (H_2O_2) has gained interest due to low health and environmental hazards, and it is considered as an "excellent and safe preservative" (Singh and Gandhi, 2015; p. 237). Hydrogen peroxide is a strong oxidizing agent and its antimicrobial activity is exerted trough the production of oxidation products which inhibit microbial proliferation (Arefin et al., 2017).

In dairy industry, the use of preservatives is crucial to obtain accurate analysis, especially in quality-based milk payment systems (Barbano et al., 2010), in which milk protein, along with fat, is included as one of the most valuable components among milk constituents, both for its influence on the cheese-making process (Visentin et al., 2017) and its importance at a nutritional level (Singhal et al., 2017). Analysis for milk recording scheme and payment systems is routinely obtained through mid-infrared spectroscopy (**MIRS**), which provides fast and reliable results. In this respect, several studies have dealt with the influence of different types of preservatives on the determination of milk components (Chalermsan et al., 2004; Zajác et al., 2016; Arefin et al., 2017) and demonstrated that the type and concentration of preservative used before analysis can influence the response of MIRS instruments (Barbano et al., 2010). Currently, HPLC is widely used as gold-standard method for qualitative and quantitative analysis of detailed milk protein profile (Niero et al., 2016; Vigolo et al., 2022). Indeed, the possibility of characterizing specific protein fractions at population level is of great interest for genetic purposes, considering the role that milk proteins exert on milk coagulation properties, cheese yield (Jõudu et al., 2008), and human health (Kay et al., 2021). Nevertheless, there is a paucity of studies that have dealt with the effect of preservative on detailed milk protein profile determined through the reference method.

Storage temperature is another factor to be addressed when considering milk chemical analysis. Ideally, milk should be analyzed immediately after sampling but, in some cases, samples are instead frozen and stored for long time before analysis. It is possible that ice crystal formations lead to the disruption of physical structures in milk (Upadhyay et al., 2014) complicating homogenization of samples and thereby underestimating target compound concentrations. To the best of the authors' knowledge, the effect of temperature and time of storage have been investigated only in relation to milk fat, protein, and CN content through MIRS (Lee et al., 1986; Barcina et al., 1987).

Therefore, the aims of the present study are (1) to determine whether the addition of different preservatives (hydrogen peroxide, Bronopol, and Azidiol), times of storage, and temperatures of analysis affect detailed milk protein composition determined by reversed-phase HPLC (**RP-HPLC**), and (2) to evaluate how and to what extent the same experimental conditions are relevant for analytical purposes in the view of their practical application.

MATERIALS AND METHODS

Experimental Design

Ethical approval was not required for the present study because cows belonged to commercial herds and milk was collected directly from the bulk tank after the milking procedure. Five raw bulk milk samples (250 mL each) were collected 3 to 5 h after morning milking on the same day of February 2021, in 5 commercial herds of primarily Holstein-Friesian breed located in the Veneto region (Italy) and associated with Latteria Soligo dairy company (Soligo, Italy). Samples were kept at cooling temperature (4°C) and immediately transported to the Department of Agronomy, Food, Natural Resources, Animals and Environment of the University of Padova (Legnaro, Italy). After accurate mixing, each bulk milk sample was divided into 5 aliquots. One aliquot (unpreserved) was delivered to the laboratory of the Breeders Association of Veneto Region (Padova, Italy) and analyzed for SCC using Fossomatic (Foss Electric A/S) according to ISO 13366–2:2006 (ISO, 2006); gross composition was determined using a MilkoScan FT7 (Foss Electric A/S) that provide information on fat, protein, CN, and lactose content according to ISO 21543:2020 (ISO, 2020). Spectral responses were routinely normalized using reference samples and reagents, following manufacturer's instructions. Both instruments worked at room temperature and samples were warmed at 37°C and homogenized by gently invert the samples 5 times before the analysis. The remaining 4 aliquots were treated each with one of 3 different preservatives, namely hydrogen peroxide (**HP**), Bronopol (**BR**), and Azidiol (**AZ**). One aliquot was used as control (no preservative; **NP**).

Preservatives were prepared as follows: (1) HP composition for 1,000 mL was sodium pyrophosphate decahydrate (0.25 g), hydrogen peroxide 35% (143 mL), and Bromophenol blue (1.75 g) in distilled sterile water; (2) BR was purchased from Knoll Pharmaceuticals (Nottingham, UK) and a 4% solution was prepared by diluting 40 g of BR (2-bromo-2-nitropropan-1,3-diol) and 0.4 g of Bromophenol blue up to 1,000 mL of distilled

sterile water; (3) AZ composition for 1,000 mL was chloramphenicol (1.5 g), ethanol (10 mL), tri-sodium citrate 5,5-hydrate (45 g), sodium azide (36 g), and Bromophenol blue (0.35 g) in distilled sterile water. Hydrogen peroxide and AZ were added to the milk aliquots at concentration of 0.25% (wt/wt), whereas BR was added at a concentration of 0.06% (wt/wt).

All the 4 differently treated aliquots were further divided into 3 subaliquots to be analyzed after different storage times as follows: no storage time (i.e., 0 d; not frozen), 7 d of storage at −20°C, and 30 d of storage at −20°C. The subaliquots were finally divided into 2 additional subaliquots to test the influence of the temperature of analysis: refrigeration temperature (5°C) and room temperature (21°C). All final aliquots (each of 500 μL) were stored in 2-mL plastic disposable tubes. Samples that had been frozen (EVERmed, LDF 925 W xPRO) reached the freezing temperature within 2 h. The experimental design is summarized in Figure 1.

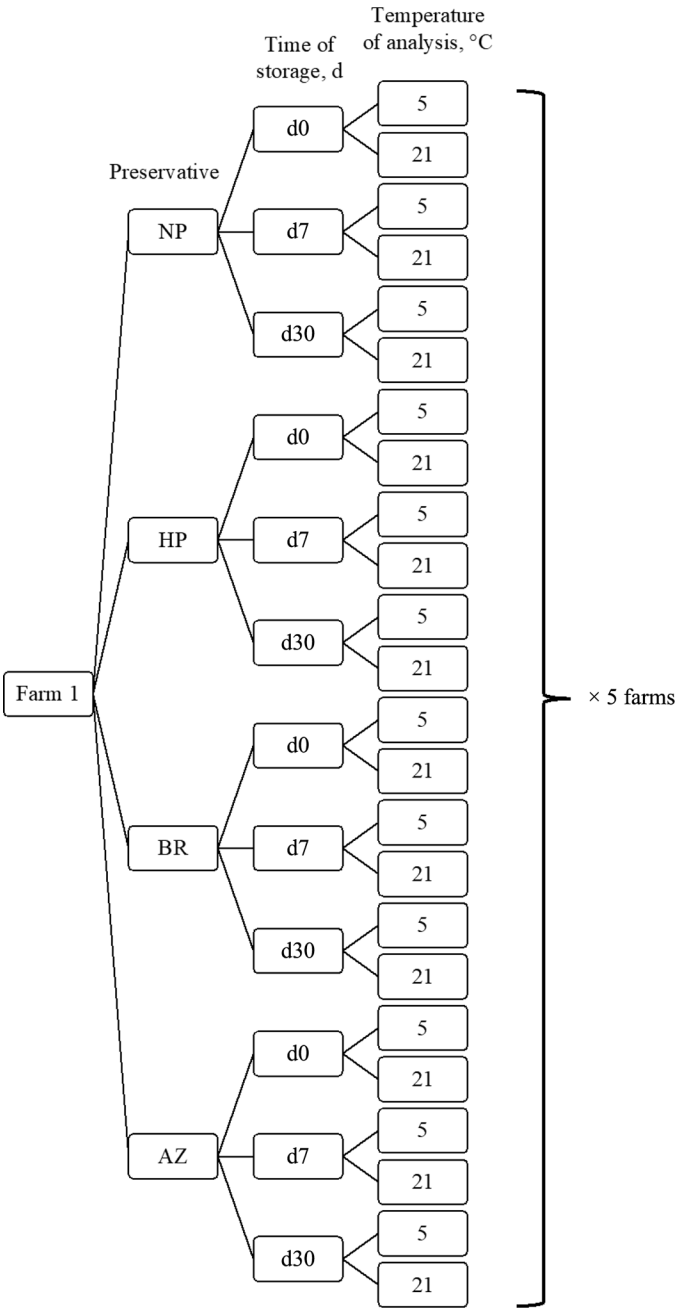

Figure 1. Experimental design. Each farm sample (n = 5) was divided into 4 aliquots and treated differently. NP = no preservative; HP = hydrogen peroxide; BR = Bronopol; AZ = Azidiol. Each preserved aliquot was divided into 3 aliquots and stored for different times: d 0 = no storage; d 7 = 7 d of storage at −20°C; d 30 = 30 d of storage at −20°C. Each stored aliquot was divided into 2 aliquots and maintained at 5°C and 21°C prior to analysis.

Chromatographic Analysis

Milk protein fractions analysis was performed through RP-HPLC. The apparatus consisted of an Agilent 1260 Infinity II LC system (Agilent Technologies) equipped with a quaternary pump (Agilent 1260 Infinity II, G7111B), a diode array detector (Agilent 1260 Infinity II, G7115A), a column thermostat (Agilent 1260 Infinity II, G7116A), and an autosampler (Agilent 1260 Infinity II, G7129A) joined to a sample cooler able to maintain the sample vial at a constant temperature by refrigerant gas (operating temperature 4–40°C). A reversed-phase analytical column C8 (Zorbax 300SB-C8 RP, Agilent Technologies) with a silica-based packing (3.5 μm, 300 Å, 150 × 4.6 i.d.) preceded by a precolumn Security Guard Cartridge System (300SB-C8 Guard Cartridges 4.6 × 12.5 mm, 4/PK, Agilent Technologies), was used for separation.

Milk samples were prepared following the method proposed by Bobe et al. (1998). Briefly, 500 μL of an aqueous solution of guanidine (Gdn) HCl (6 *M* GdnHCl, 0.1 *M* BisTris buffer, 5.37 m*M* sodium citrate, and 19.5 m*M* DTT) were added to milk in a 1:1 ratio (vol/vol). The solution was added directly to frozen aliquots at room temperature. Each sample was vortexed for 10 s, incubated at room temperature for 1 h to promote protein solubilization, and centrifuged for 10 min at room temperature at 13,000 × *g* to promote the separation of fat. The soluble phase was added to a solution containing 4.5 GdnHCl diluted in a solvent consisting of water, acetonitrile, and trifluoroacetic acid (100:900:1; vol/vol/vol), in a proportion of 1:3 (vol/vol). The separation of milk protein fractions was conducted following the method proposed by Bonfatti et al. (2008). Gradient elution was carried out with a mixture of 2 solvents: solvent A (0.1% trifluoroacetic

acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile). Separations were performed with the following gradients: linear gradient from 33 to 35% B in 5 min, from 35 to 37% B in 4 min, from 37 to 40% B in 9 min, from 40 to 41% B in 4 min, isocratic elution at 41% B for 5.5 min, linear gradient from 41 to 43% B in 0.5 min, and from 43 to 45% B in 8 min. Before the injection of the succeeding sample, the column was re-equilibrated at 33% B for 8 min. The total analysis time per sample was 44 min. The flow rate was 0.5 mL/min, the column temperature was kept at 45°C, the detection was made at a wavelength of 214 nm, and the injection volume was 5 μL. Agilent OpenLab 2 CDS software (Agilent Technologies) was used for data acquisition and analysis. Identification and quantification of different milk protein fractions were carried out using external standards of α-CN, β-CN, κ-CN, α-LA, β-LG A, and β-LG B (Merck KGaA) at the highest available purity level. Quantification of each chromatographic peak was obtained with 5-point calibration curves ($R^2 \geq 0.99$).

Statistical Analysis

The concentration of each protein fraction was adjusted, accounting for the dilution effect due to different volumes of preservatives added to the milk samples. Sources of variation were investigated using the MIXED procedure of SAS software v. 9.4 (SAS Institute Inc.), according to the following linear mixed model:

$$y_{ijkl} = \mu + P_i + S_j + T_k + (P \times S)_{ij} + farm_l + \varepsilon_{ijkl},$$

where y_{ijkl} is the dependent variable [amount of total CN, α_{S1}-CN, α_{S2}-CN, β-CN, κ-CN, total whey proteins (**WP**), β-LG, or α-LA]; μ is the overall intercept of the model; P_i is the fixed effect of the ith type of preservative (i = NP, HP, BR, AZ); S_j is the fixed effect of the jth time of storage (j = 0, 7, 30 d at −20°C); T_k is the fixed effect of the kth temperature of autosampler (k = 5°C, 21°C); $(P \times S)_{ij}$ is the fixed interaction effect between type of preservative and time of storage; $farm_l$ is the random effect of the lth farm (l = 1–5); and ε_{ijkl} is the random residual. A multiple comparison of least squares means for the fixed effects was performed using Bonferroni's test ($P < 0.05$).

To evaluate the similarity between detailed protein profile measured under different experimental conditions and detail protein profile of NP milk, the z-score (z) was calculated as the following:

$$z = \frac{m - VAL_{REF}}{SD},$$

where m is the average of each protein fraction of NP samples analyzed at 0 d at 5°C; VAL_{REF} and SD are the median and the standard deviation, respectively, of each protein fraction calculated for each combination of fixed effects included in the model, for a total of 23 theses with 5 observations each (Figure 1). Results from 2 experimental conditions are considered equal when $|z| \leq 2$, similar when $2 < |z| \leq 3$, and different when $|z| > 3$ (Thompson et al., 2006).

RESULTS AND DISCUSSION

Bulk Milk Composition

Descriptive statistics of bulk milk chemical composition and SCC are reported in Table 1. Milk fat, protein, casein, and lactose averaged 3.97, 3.39, 2.70, and 4.76%, respectively, and the coefficient of variation (**CV**) varied from 0.84% (lactose) to 4.28% (fat). Average milk chemical composition was comparable to that observed in previous studies on Italian bulk milk (Penasa et al., 2016; Benedet et al., 2018). The SCC averaged 234.00×10^3 cells/mL and exhibited the greatest CV (36.01%). Even if SCC had the greatest CV, the variation is relatively low; this can be explained by a certain homogeneity of herds included in the study in terms of geographical area, sampling period, management, and breed. Also, it is worth noting that we considered bulk milk samples, which again can explain the low variability of the considered trait. Indeed, this selection was purposely made to minimize external sources of variation.

With respect to detailed milk protein fractions, α-CN (as sum of α_{S1}-CN and α_{S2}-CN) and β-CN accounted for 82% of the total milk casein content, the remaining part being represented by κ-CN, with an average value of 5.84 mg/mL (Table 1). Whey proteins β-LG and α-LA averaged 4.28 and 1.21 mg/mL, respectively; the α-LA was the least abundant protein fraction but exhibited the greatest CV (9.72%). Vigolo et al. (2022) applied the same chromatographic method for the quantification of milk protein fractions in bulk milk samples and obtained similar results for α_{s1}-CN, κ-CN, β-LG, and α-LA, slightly lower concentration of α_{S2}-CN, and greater concentration of β-CN.

Total protein content calculated as the sum of total casein and total WP, was greater compared with the same trait obtained through MIRS prediction. Indeed, as reported by Niero et al. (2016), values obtained through HPLC technique can be overestimated compared with other quantification methods. One possible explanation is the presence of contaminants in the external standard used for calibration; when each standard is weighed, part of the weight is buffering salt

Table 1. Descriptive statistics of untreated raw bulk milk composition collected in 5 farms (n = 5)

Trait	Mean	SD	CV, %	Minimum	Maximum	Range
Milk composition						
Protein, %	3.39	0.08	2.35	3.25	3.48	0.23
Casein, %	2.70	0.07	2.59	2.56	2.78	0.22
Fat, %	3.97	0.17	4.28	3.81	4.23	0.42
Lactose, %	4.76	0.04	0.84	4.71	4.80	0.09
SCC, $\times 10^3$ cells/mL	234.00	84.27	36.01	108.00	361.00	253.00
Protein fraction, mg/mL						
Total CN	34.80	1.46	4.19	32.84	36.34	3.50
κ-CN	6.21	0.44	7.08	5.55	6.69	1.14
α_{S1}-CN	12.39	0.46	3.71	11.75	12.91	1.16
α_{S2}-CN	5.50	0.47	8.54	4.93	6.00	1.07
β-CN	10.70	0.32	2.99	10.21	10.94	0.73
Total whey protein	5.98	0.19	3.18	5.75	6.26	0.51
α-LA	1.38	0.05	3.62	1.32	1.44	0.12
β-LG	4.60	0.17	3.70	4.44	4.88	0.44

used to isolate the protein standard. The percentage of salt is likely to vary according to protein standard (i.e., from one batch to another) and across different protein standards (i.e., from one standard protein fraction to another). This might distort the estimation of the ratios of the different fractions obtained through the chromatographic method. Accordingly, certificates of analysis of the external standard used for calibration attested a protein content of less than 100%. Nevertheless, because the available studies dealing with chromatographic determination of protein fraction employ commercial standards at highest available purity level without further adjustment, we decided not to correct the weight of the standard to make results comparable.

Factors Affecting Casein Profile

Results from the ANOVA for CN fractions are summarized in Table 2. The storage time and the type of preservative affected significantly all the CN fractions ($P < 0.01$) except for α_{S2}-CN. On the contrary, the interaction between type of preservative and storage time only affected β-CN ($P < 0.05$). The temperature of analysis did not affect the total amount of CN but significantly contributed to explain the variation of κ-CN, β-CN($P < 0.01$), and α_{S1}-CN ($P < 0.05$). The total variance accounted for by the random effect of herd ranged from 27.93 to 78.83% for α_{S2}-CN and κ-CN, respectively.

The least squares means of detailed milk protein fractions for the different types of preservative are reported in Table 3. The NP samples had greater α_{S1}-CN and κ-CN content than samples with preservative ($P < 0.05$). Also, β-CN content was greater ($P < 0.05$) in NP samples than in BR-preserved samples. Bronopol's high activity against gram-negative bacteria is due to its ability to release formaldehyde. The lower amount of CN of BR-preserved samples could partially be explained by the fact that formaldehyde reacts with amino groups of milk protein, creating formal-protein complexes (Upadhyay et al., 2014). Because the use of BR is widespread in dairy laboratories, numerous

Table 2. *F*-values and significance of fixed effects included in the model for detailed milk protein composition

	Fixed effect				Random effect	
Trait[1]	Preservative	Storage time	Temperature of analysis	Preservative × Storage time	σ^2_{farm},[2] %	RSD[3]
Total CN	4.36**	38.78***	3.86	1.61	53.93	1.00
κ-CN	9.57***	37.22**	12.64**	0.94	78.83	0.20
α_{S1}-CN	9.42***	55.96***	6.30*	0.94	50.13	0.34
α_{S2}-CN	2.19	1.25	2.00	1.19	27.93	0.35
β-CN	4.07**	58.28***	9.32**	2.39*	44.64	0.29
Total WP	12.57***	18.04***	24.04***	1.27	35.36	0.23
α-LA	9.63***	15.13***	3.24	1.44	36.59	0.17
β-LG	11.49***	21.04***	33.59***	1.30	42.90	0.08

[1]WP = whey protein.
[2]σ^2_{farm} = proportion of total variance accounted by farm effect.
[3]RSD = residual standard deviation.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 3. Least squares means of detailed milk protein composition for different types of preservative

Trait	Preservative			
	No preservative	H_2O_2	Bronopol	Azidiol
Protein fractions, mg/mL				
Total CN	33.46[a]	33.03[ab]	32.53[b]	32.92[ab]
α_{S1}-CN	11.89[a]	11.60[b]	11.44[b]	11.56[b]
α_{S2}-CN	5.34[a]	5.52[a]	5.38[a]	5.53[a]
β-CN	10.22[a]	10.07[ab]	9.98[b]	10.03[ab]
κ-CN	6.00[a]	5.85[b]	5.73[b]	5.80[b]
Total whey protein	5.62[a]	5.57[a]	5.29[b]	5.49[a]
β-LG	4.36[a]	4.34[a]	4.13[b]	4.29[a]
α-LA	1.26[a]	1.23[ac]	1.16[b]	1.19[bc]

[a–c]Least squares means with different superscript letters within a row are significantly different ($P < 0.05$).

studies have dealt with its influence on routine analysis, mainly through MIRS, and contrasting results have been reported. Lee et al. (1986) observed higher protein levels in BR-preserved than in untreated cow milk samples, and the same findings were reported by Sánchez et al. (2005) in goat milk. Conversely, Sešķēna and Jankevica (2007) concluded that the addition of 0.04% BR did not affect the milk protein content. Such results could be explained by the fact that it tested the combined effect of all the ingredients composing the preservatives, and not the separate effect of each single component that, instead, could have its own separate effects on one analytical method versus another. To the best of the authors' knowledge, the present study is the first dealing with the effect of BR on protein profile measured through RP-HPLC.

As regards HP and AZ, their addition to milk had a negative effect especially on κ- and α_{S1}-CN, with significantly less content as compared with NP samples. Nevertheless, the magnitude of such effect did not influence the overall amount of total CN in agreement with Sešķēna and Jankevica (2007), who did not observe a strong effect of HP and AZ on MIRS-predicted milk total protein content. Accordingly, Barcina et al. (1987) concluded that AZ does not modify the protein content of milk samples and is suitable for MIRS analysis. Again, to the authors' knowledge, there are no studies on protein analysis through RP-HPLC to be used as a comparison with the results of the present study.

Least squares means for different days of storage and different temperatures of analysis are presented in Table 4. The κ-CN, β-CN, and α_{S1}-CN showed significantly greater contents ($P < 0.05$) when analyses were performed at 5°C (refrigeration temperature) compared with 21°C (room temperature). The greatest amount of CN fractions was observed in samples that were not frozen, i.e., samples analyzed at 0 d. Indeed, except for α_{S2}-CN, all protein fractions underwent a significant decrease at d 7 ($P < 0.05$). Accordingly, it was extensively reported that the freezing process can affect the final quality of milk protein and CN fractions mainly due to the growth of ice crystals and the formation of separated phases. This may result in damage to the matrix and biased sampling during analytical procedures (Koschak et al., 1981; Alinovi et al., 2021). The storage temperature of −20°C (slow freezing) is the temperature conferring greater protein stability, whereas lower temperatures (e.g., −78°C, rapid freezing) cause an unfavorable environment for proteins (Koschak et al., 1981). The effectiveness of slow freezing was confirmed also by our results, where the total CN content decreased by 3.67% after 7 d of storage and by 5.73% after 30 d. These data portray a gradual, nonlinear negative effect of storage time on CN fraction (longer storage time causes a greater decline). The freezing procedure, in fact, guaranteed a more stable matrix with a tendency for the decline of the total CN to decelerate: as mentioned above, the observed decrease was almost 4% after 7 d of storage (from 34.05 to 32.80 mg/mL) but the percentage of decrease halved after 30 d of storage (2.13%). This constant nonlinear pattern of deterioration described for total CN was also observed for β-CN which showed a decrease of 4.11% after 7 d of storage and of 6.60% after one month, confirming the effect freezing temperatures exert on the deceleration of degradation of protein fractions. The κ-CN demonstrated the most stable behavior by not undergoing further degradation after 7 d of storage. On the contrary, α_{S1}-CN was the fraction affected most by storage time, experiencing a linear degradation over time.

Factors Affecting WP Profile

Results reported in Table 2 highlight that almost all the effects included in the model, except for the interaction between type of preservative and storage

Table 4. Least squares means of detailed milk protein composition for different storage times and temperatures of HPLC autosampler

	Storage time[1]			Temperature of analysis	
Trait	0 d	7 d	30 d	21°C	5°C
Protein fractions, mg/mL					
Total CN	34.05[a]	32.80[b]	32.10[c]	32.80[a]	33.16[a]
α_{S1}-CN	12.03[a]	11.62[b]	11.22[c]	11.54[b]	11.70[a]
α_{S2}-CN	5.51[a]	5.43[a]	5.39[a]	5.49[a]	5.40[a]
β-CN	10.45[a]	10.02[b]	9.76[c]	9.99[b]	10.15[a]
κ-CN	6.07[a]	5.72[b]	5.74[b]	5.78[b]	5.91[a]
Total whey protein	5.66[a]	5.45[b]	5.36[b]	5.39[b]	5.59[a]
β-LG	4.40[a]	4.29[b]	4.15[c]	4.19[b]	4.37[a]
α-LA	1.26[a]	1.16[b]	1.21[b]	1.20[a]	1.22[a]

[a–c]Least squares means with different superscript letters within row and effect are significantly different ($P < 0.05$).

[1]0 d of storage = fresh sample; 7 and 30 d of storage at −20°C.

time, contributed to the variation of WP fractions ($P < 0.001$). For total WP fraction, the random effect of herd accounted for more than 35% of the total phenotypic variance.

The β-LG and α-LA presented the same pattern of CN fractions, with greater values for NP samples and lower for BR-preserved samples ($P < 0.05$; Table 3). Among all the preservatives, BR was the only one leading to significantly lower total WP content ($P < 0.05$) compared with other theses. Regarding HP treatment, at high H_2O_2 concentration, β-LG tends to break down into low molecular weight components (Luck and Joubert, 1955) but values of HP-preserved samples neither demonstrate any significant decrease in β-LG fraction nor in total WP confirming that the concentration of H_2O_2 adopted is optimal for milk intended for analysis. The addition of AZ for the preservation of the sample is still common because it combines antibacterial agents and bacteriostatic antibiotics (chloramphenicol and sodium azide, respectively; Upadhyay et al., 2014), ensuring a wide spectrum of action. The AZ preservative had an effect only on α-LA fraction with a value 5.88% lower than that of NP samples ($P < 0.05$) but, overall, total WP amount in samples preserved with AZ did not differ significantly from NP samples and HP-treated samples.

Storage time was also a non-negligible source of variation. Least squares means confirm that freezing temperature affected detailed milk protein profile: after 30 d of storage, total WP decreased from 5.66 to 5.36 mg/mL ($P < 0.05$; Table 4). Nevertheless, looking at the 2 WP fractions in detail, it is possible to observe 2 contrasting behaviors: β-LG was significantly affected by storage time ($P < 0.05$), undergoing a linear degradation of 2.50% after 7 d of storage and 5.68% after 30 d, whereas α-LA decreased only until 7 d of storage.

Temperature of analysis had greater effect on WP compared with total CN; indeed, the 2 tested temperatures determined a significant difference on total WP ($P < 0.05$; Table 4). Particularly, the refrigeration temperature (5°C) preserved more β-LG compared with environmental temperature (21°C). This confirms the discrepancy between results obtained with chromatographic stations that keep samples at a constant refrigeration temperature prior to analysis and those obtained at room temperature.

Practical Implications

Least squares means presented in the previous paragraphs aimed to assess the parameters at which detailed milk protein composition is statistically affected by different preservatives, storage times, and temperatures of analysis. It is not clear if such differences are significant also from a practical point of view. For this reason, z-scores were calculated as pairwise comparison between distributions of 5 observations each, to evaluate whether κ-CN, α_{S1}-CN, α_{S2}-CN, β-CN, α-LA, β-LG, total CN, and total WP determined in NP milk measured at 0 d and 5°C, agreed with the same traits determined in milk treated according to the 23 experimental conditions tested (Table 5 and Figure 1). Usually, results from 2 experimental conditions may be considered equal when $|z| \leq 2$, similar when $2 < |z| \leq 3$, and different when $|z| > 3$ (Thompson et al., 2006).

Among all protein fractions, β-LG was the most stable across different conditions, with z-score averaging 0.44 and ranging from 0.01 to 1.23 in absolute values. Similar results were obtained for α_{S2}-CN fraction, with z-score averaging 0.60 and being >2 only for milk added with AZ analyzed on d 30 at 21°C. The κ-CN exhibited slightly greater average z-score (0.94),

Table 5. Z-scores[1] calculated on detailed milk protein composition according to different preservatives, storage times, and temperatures of analysis

Preservative[2]	Storage time,[3] d	Temperature of analysis, °C	κ-CN	α_{S1}-CN	α_{S2}-CN	β-CN	α-LA	β-LG	Total CN	Total whey protein
NP	0	5	—	—	—	—	—	—	—	—
NP	0	21	0.39	0.37	0.35	0.35	0.54	0.13	0.65	0.36
HP	0	5	0.51	0.36	0.05	0.52	2.34	0.28	0.99	0.45
HP	0	21	0.50	1.80	0.00	0.98	0.80	0.27	1.42	0.92
BR	0	5	0.02	0.33	0.61	0.77	1.78	0.05	0.38	0.27
BR	0	21	0.68	2.20	0.47	1.66	1.61	0.69	2.89	2.23
AZ	0	5	0.18	0.50	0.78	0.54	2.30	0.01	0.31	0.47
AZ	0	21	1.05	2.16	0.74	2.09	1.74	0.30	3.80	2.45
NP	7	5	0.53	0.76	0.62	2.21	1.92	0.16	2.15	1.41
NP	7	21	0.77	0.79	0.00	1.84	1.60	0.07	1.62	0.96
HP	7	5	1.63	2.73	0.02	2.66	1.72	0.54	1.45	2.42
HP	7	21	1.46	1.38	0.45	1.65	1.89	0.17	1.90	1.90
BR	7	5	1.42	2.70	0.07	3.16	2.37	1.00	1.65	1.82
BR	7	21	1.63	1.69	0.77	2.87	2.61	0.94	1.45	1.88
AZ	7	5	0.98	0.91	0.75	1.88	1.81	0.38	2.20	0.96
AZ	7	21	1.43	1.85	0.53	2.40	2.25	0.26	0.52	1.65
NP	30	5	0.96	1.27	0.93	1.63	1.78	0.37	2.07	1.39
NP	30	21	0.66	1.98	0.53	2.80	2.28	0.65	3.65	2.44
HP	30	5	1.23	4.47	0.70	1.71	0.59	0.21	0.88	1.69
HP	30	21	1.03	3.07	0.96	4.77	1.56	0.73	4.35	3.89
BR	30	5	0.43	1.99	0.93	1.55	1.05	0.41	2.11	1.09
BR	30	21	1.77	3.90	0.08	5.45	3.50	1.23	4.36	4.16
AZ	30	5	1.00	3.98	1.03	2.62	1.35	0.48	2.82	2.48
AZ	30	21	1.39	3.88	2.52	3.96	2.75	0.82	4.32	3.39

[1]Results from 2 experimental conditions are considered equal when $|z| \leq 2$, similar when $2 < |z| \leq 3$, and different when $|z| > 3$.
[2]NP = no preservative; HP = hydrogen peroxide; BR = Bronopol; AZ = azidol.
[3]0 d of storage = fresh sample; 7 and 30 days of storage at −20°C.

but values were always <2. In the light of the results of the present study and from a practical point of view, we can support that β-LG, α_{S2}-CN, and κ-CN are not affected by different preservatives, storage times (up to 30 d at −20°C), and temperatures of analysis (up to 21°C).

The α-LA and α_{S1}-CN fractions exhibited similar z-scores, with average values of 1.83 and 1.96, respectively. In the case of α-LA, z-scores were >3 in 0.5% of the tested theses, comprised between 2 and 3 in 30% of the tested theses, and lower than 2 in the remaining cases. The α_{S1}-CN showed a higher incidence of z-scores >3 (22% of the tested theses) and lower frequency of z-scores between 2 and 3 (17% of the tested theses). Based on the average z-score, we can support that the quantification of both α-LA and α_{S1}-CN are affected to merely a negligible extent by different experimental conditions. Still, particular attention must be placed on the storage time, which should be limited to avoid significant deviations in the profiling of these 2 protein fractions.

The β-CN exhibited the greatest average z-score (2.18), being >3 and between 2 and 3 in 17% and 30% of the tested theses, respectively. Also, in this case, short storage time should be preferred over long storage time as it allows for minimal degradation, and thereby underestimation, of the β-CN fraction.

CONCLUSIONS

The present study aimed to assess the effect of different preservatives, storage time, and temperature of analysis on detailed milk protein composition determined through RP-HPLC analysis. Results revealed that samples without preservatives had significantly greater amount of all protein fractions. Among the tested preservatives, BR was associated with the lowest concentrations of protein fractions, probably due to the development of complexes between formaldehyde and proteins. As expected, increasing time under freezing conditions had a detrimental effect on milk protein composition. From a practical point of view and in respect to routine laboratory analyses, z-scores suggested a general agreement between detailed protein profile determined on treated milks compared with detailed protein profile of fresh milk without preservative. Significant deviations were observed only in correspondence with long storage time under freezing conditions, which therefore should be avoided to obtain reliable results.

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