Biointerface Research in Applied Chemistry

Method validation and determination of hydroxymethyl furfural (HMF) and furosine as indicators to recognize adulterated cow's pasteurized and sterilized milks made by partial reconstitution of skim milk powder

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ABSTRACT

In this research, Hydroxymethylfurfural (HMF) and Furosine, the two important markers of Maillard reaction, were determined as potential indicators in order to identify the adulteration of skim milk powder (SMP) addition (instead of milk solid non-fat) for the formation of cow's milk (Pasteurized and sterilized). Method validation was performed by HPLC. Pasteurized and sterilized milks (2.5% fat) with 25, 50 or 75% reconstitution of milk powder were produced. By using HPLC method, there was a significant relation between HMF and furosine contents and an increase of SMP content. The correlation between the amount of added powder and HMF or furosine concentration was almost linear. Thus, the amount of SMP addition could be determined by quantification of HMF and furosine. Sensory evaluation and optical properties of treatments were also assessed. The sensory acceptance was significantly decreased by increasing SMP substitution. Colorimetric evaluation did not show a significant difference between the treatments. The intera-day and inter-day relative standard deviation (RSD%) was 5.9% and 4.1% for HMF and furosine, respectively. The recovery rates were 85% and 91% and the LOD values for HMF and furosine were 0.05 and 0.02 ppm, respectively. The markers were realized effective and applicable for detecting SMP addition adulteration in pasteurized and sterilized cow's milk.

Keywords: *Adulteration, milk powder, furosine, hydroxymethyl furfural (HMF), HPLC, method validation, sensory evaluation.*

1. INTRODUCTION

According to FAO statistics (2015), 85% of total milk production comes from cows, followed by buffaloes (11%), goats (2%), sheep (1%) and camels (0.4%). Dairy industry has a very important role in the food industry with a considerable value of 15%. Cow's milk was one of the 20 most important food and agricultural commodities in the world in 2015 and cheese, yogurt, liquid milk and milk powder were consumed as milk products [1]. Pasteurized and sterilized milk produced by dairies must be prepared from original fresh milk. However, producing adulterated fresh milk by partially or completely addition of milk powder (partial or complete substitution of milk solid non –fat with SMP) without specifying on label of the product may have economic benefits and be occurred. Therefore, the detection of milk powder in adulterated milks is currently an important practical problem with industrial and governmental aspects.

Reactions such as non-enzymatic glycosylation (Maillard reaction), sugar isomerization, and whey protein denaturation occur during thermal treatment of milk. The non-enzymatic reaction between reducing sugars and free amino group of proteins leads to the glycated proteins termed 'Amadori products'. Formation of some components (e.g., hydroxymethyl furfural (HMF), furosine and lactulose or degradation of original constituents (e.g. lactoglobulin) can be regarded as potential indicators to show the severity of thermal processes as well as the Maillard reaction [2, 3]. HMF is formed in the Maillard reaction as well as during caramelization. It is also generated slowly during the storage of thermally processed foods [4-6]. Furosine is formed during acid hydrolysis of the Amadori compound fructosyl-lysine, lactulosyllysine and maltulosyl-lysine, produced by the reaction of ε-amino groups of lysine with glucose, lactose and maltose, respectively [7, 8]. Therefore, it has been used to measure the early stages of Maillard reaction in biological samples along with foods [9]. Various process conditions especially heat treatments result in the formation of different extents of HMF and furosine [10, 11]. As heat-treated milk products (e.g., pasteurized milk, sterilized milk and skim milk powder or SMP) are heated in different degrees from mild to severe, we hypothesized that the rates of significantly different produced HMF and furosine in these products could be correlated to the source of product, and to the amount of milk powder added [12].

There are some researches regarding the determination of added SMP into dairy products. Abasyanic et al. (2016) optimized an analytical HPLC method to qualify and quantify milk powder in UHT and pasteurized milk [13]. Furosine has been used as an

indicator of milk powder. It has been accepted a linear correlation between the milk powder and furosine concentrations. Guan et al. (2005) and Liu et al. (2016) developed a FAST (fluorescence of advanced Maillard products and soluble Tryptophan) method for identification of reconstituted milk made from skim milk powder in the fresh milk and showed differences between raw and reconstituted milk [14, 15]. Madkur et al. (1989) determined the adulteration of fresh milk with reconstituted full fat milk powder [16]. The ultraviolet and visible spectra (240 to 700 nm) indicated two empirical parameters used to detect and quantify adulteration. The sensitivity was 2.5% for added reconstituted milk powder. The effect of low fat values (less than 2.3%) was discussed and a correction factor derived. Ohta et al. (2002) examined the use of furosine as an index for the detection of reconstituted skim milk in cow's milk [17]. Furosine contents were quantitatively determined using high performance liquid choromatography (HPLC). Furosine concentration was observed to be highly dependent on the reconstituted skim milk concentration, and linearly increased with an increase in the percentage of reconstituted skim milk to genuine milk. The results of the quantitative analysis of furosine in commercial UHT-milk products showed that milk products containing reconstituted skim milk had much higher furosine than genuine milk UHT products.

Rehman et al. (2000) showed that the detection of dried milk powder in liquid milk was based on the determination of (HMF) values [18]. The HMF in raw unheated milk was 7.66 µ mol/L which in the pasteurized and UHT milk, respectively, ranged between 10.52-16.0 µ mol/L and 16.33-20.85 µ mol/L. The HMF in dried milk powder reconstituted to liquid milk with SNF 9% was 28.0 µ mol/L.

The aim of this study was to validate method for determination of the two principal markers of Maillard reaction in heated milk and milk products (hydroxymethyl furfural and furosine) in pasteurized and sterilized milk adulterated with addition of SMP as a partial replacer of fresh milk dry matter. Also, to find a correlation between the amount of mentioned markers and the concentration of replaced SMP.

2. MATERIALS AND METHODS

2.1. Reagent, materials and standards.

Hydroxymethyl furfural was purchased from Sigma–Aldrich (Steinheim, Germany) at purity higher than 99% and furosine was purchased from Carbosynth Co. (Yorkshire, England). 1-Octanol, ethanol, acetic acid, ammonium phosphate, and sodium 107 chloride (analytical grade), sodium acetate, glacial acetic acid, acetonitrile, HCl and water (HPLC-grade) were obtained from Merck (Darmstadt, Germany). Pasteurized and sterilized milk samples (2.5% fat) from five brands (Tehran market, Iran) and skim milk powder (dry matter of 97.9%) (Pegah Co., Tehran. Iran) were prepared.

2.2. Instrumentation.

HPLC (UV-detector, Agilent), HPLC software (LC solution), microfilters (RC 0.45 µ), analytical balance (Sartorius CP 323S, Guttingen, Germany), oven (Nuve, Menominee, America), sealing vials (glass vials), paper filters and glass syringe (Hamilton, Switzerland) were used. Pre-wetted Sep-pak C18 cartridge (Waters Co., USA) supernatant was used to HPLC after loading. Spherisorb column ODS2 (octadecylsilane) 5 µm column (0.46×25cm, Waters Co., USA) A C18 column (Therma Scientific ODS Column 250-4.6-5), was used for HPLC determination. The volume of injection was 20 µL, the detector wavelength was 284 nm, the acetate buffer was separated (0.2 mol/L, pH = 3) and the acetonitrile column (85:15) was washed with a flow rate of 0.8 mL/min.

A combination of acetonitrile (15%) and sodium acetate buffer (85% PH = 3) were considered as HPLC solvent. The furosine and HMF were measured by HPLC with a UV-Vis detector with a wavelength 284 nm, which is equivalent to max λ. The sensitivity of the HPLC device was set at 0.005 and the injection volume was 20 mL. With the washing program mentioned in the materials and equipment section, these analytes were measured. Chromatograms of injection 20 µL milk solution containing furosine and HMF are shown in Figure 1. Retention time of HMF was about 5.5 min and for furosine, it was about 6.5 min.

2.3. Sample preparation for HPLC analysis.

Initially, hydroxymethyl furfural and furosine standard solutions were prepared at 2000 ppm and 1000 ppm, respectively, in dark-colored vials. Subsequently, the dilutions (1:3) were performed to meet the standards of this metabolite at values of 20, 10, 5, 1, 0.5 and 0.1 ppm by adding to diluted cow's milk. Prepared samples were stored at refrigerator temperature. The chemicals used in this project were furosine (Carbosynth, England) and HMF (Sigma Aldrich, Germany) with a purity of ≥ 98.0% (GC/HPLC), acetone buffer (HPLC grade), pH = 3, 85% of mobile phase (Merck, Milipore), and acetonitrile (HPLC grade) with a 0.2% concentration of 15% Milipore mobile phase (Deionized water, purification system Q-Cheek USA).
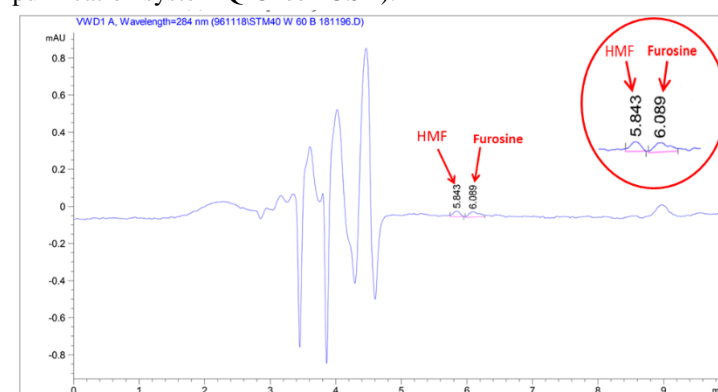
Figure 1. Chromatogram of extraction and determination of furosine and HMF in commercial milk samples.

2.4. Method validation.

Linearity, selectivity, accuracy (recovery), precision, LOD, and LOQ were determined according to AOAC guideline [19]. These parameters were specified for validation of the method of HMF and furosine determination in milk samples. The validation of

HPLC-UV method for the determination of extracted HMF and furosine in milk was carried out. Calibration of method for milk matrix was performed by spiking concentrations from 0.1 to 10 μg/mL (ppm) of HMF and furosine to diluted cow's milk with water by a ratio of 1:3. The linearity of the method was examined by analyzing the concentrations ranging from 0.1 to 10 ppm in spiked diluted cow's milk.

Recovery and precision (expressed as %RSD) were evaluated by analyzing samples spiked with the stock solution to final concentrations of 0.5, 2.5, or 10 ppm. Intra-day precision (3 repetitions on the same day) and inter-day precision (5 repetitions over three altered days) tests were achieved. Finally, the uncertainty measurement during each step of the analytical procedure was calculated by the bottom-up method from the validation data [19].

2.5. Dispersive liquid-liquid microextraction (DLLME).

Extraction solution containing 650 mL of ethanol (as a disperser solvent) and 60 mL 1-octanol (as an extraction solvent) were added immediately to 9 mL milk sample and then the mixture was shaken severely for 2 min. In this step, the HMF in the milk sample is extracted into 1-octanol and the solution color would change to the filmy. Then, the solution was centrifuged at 1789 g for 2 min, 40 mL 1-octane phase was separated from the aqueous phase, and 20 mL of the underlying blue phase was injected into HPLC [20, 21].

2.6. Determination of HMF and furosine.

1 mL sample solution was hydrolyzed through the addition of 8 ml of 8 N HCl at 110°C. It was placed in a flooded tube for 23 h. During this time, the nitrogen gas passed through the solution and the hydrosolized product was dried and dissolved in 0.5 mL water. The solution was injected into a pre-wetted Sep-pak C18 cartridge and the washing program of 5 mM sodium heptanesulphonate with 20% acetonitrile as organic modifier and 0.2% formic acid and flow rate of 2.81 mL/min was used [22].

2.7. Milk samples preparation.
Pasteurized and sterilized milk samples (2.5% fat content) prepared from one batch number of a commercial brand (Pegah Co., Tehran, Iran) with determined dry matter were diluted to definite extents followed by adding certain concentrations of SMP in order to achieve pseudo-adulterated samples with 25, 50 or 75% replacement of milk solid non-fat with SMP. The resulted samples were kept refrigerated until used for analysis of furosine and HMF.

2.8. Optical determination.

Hunter Lab method was used for the colorimetry of milk samples [23]. The Hunter system of color measurement was more sensitive to differences in whiteness among milk samples. The lightness (L), redness (a), and yellowness (b) color system was used to evaluate the color of treatments by a colorimeter (ColorFlex EZ Spectrophotometer, Hunter Associates Laboratory, Inc). The measurements were taken on white standard backgrounds (L* = 93.49, a* = −0.25 and b* = −0.09). Total color difference (ΔE) and whiteness index (WI) were calculated using the following equations:

$$\Delta E = \sqrt{(L*-L_0)^2 + (a*-a_0)^2 + (b*-b_0)^2}$$

$$WI = 100 \; - \sqrt{(100-L)^2 + a^2 + b^2}$$

Where, L*, a*, and b* are the color parameter values of the standard and L, a, and b are the color parameter values of the sample.

2.9. Sensory evaluation.

Sensory evaluation test was carried out using different test with a 5-point scale (1= unrealized and 5= intense) for cooked and powdery flavors and a 3-point scale for color (1= regular milky and 3= turbid milky). 25 trained assessors were used for analysis.

2.10. Statistical analysis.

All the experiments and analysis were performed in triplicate and the means were presented as means ± standard deviation (SD). Statistical analysis of data was performed by one-way analysis of variance and Tukey's test using the SPSS 21.0 software package program. Values of $p < 0.05$ were regarded as significant.

3. RESULTS

3.1. Method validation.

The analytical parameters of method validation are presented in Table 1. Calibration curve could be used to measure the composition of milk by plotting the area of the HPLC peak for different milk percentages. The resulted calibration curves by linear regression were linear with the correlation coefficient of 0.992 and 0.988 for HMF and furosine, respectively. By comparing the slope of the furosine equation, it showed higher sensitivity and precision than HMF calibration curve. For furosine and HMF equation, the determination of HMF had lower sensitivity than furosine sensitivity (Figure 2). The linearity in the studied samples was reliable. LOD values were 0.05 and 0.02 for HMF and furosine, respectively. We could not find any study showing LOD and LOQ for HMF and furosine by using HPLC method. However, Bignardi et al. (2012) investigated furosine in some foods such as pasta, milk, and tigelle bread by using capillary electrophoresis coupled to tandem mass spectrometry and HPLC and reported values of 0.07 and 0.25 mg L^{-1} for LOD and LOQ, respectively [24]. In this association, Morales et al. (2000) used a HPLC method in order to determine the HMF in industrial processed milk [25]. They digested samples with oxalic acid (0.3 N) for 1 h at 100°C, deproteinized with trichloroacetic acid solution (40%, w/v), and filtered prior to the HPLC determination with detection at 280 nm. The detection limit of the method was 0.2 μmol/L. In a study by Schmidt et al. (2017), LOD and LOQ in determination of furosine in commercial milk samples by ultra-high performance liquid chromatography were reported as 150 nmol/L and 45 nmol/L, respectively [26].

3.2. Recovery and precision.

Our findings for recovery and precision for the proposed determination method for concentrations of 0.5, 2.5 and 10 ppm are shown in Table 2. Inter-days RSD and intraday RSD were

decreased and recovery was increased with increasing concentration from 0.5 up to 10 ppm. The mean recovery values ranged from 73% to 89% for HMF at 0.50 up to 10 ppm. However, the data were 84% up to 94% for furosine. Regarding HMF, the data for inter-days RSD was between 7.5 to 12.5 and 3.8 to 12.50 for intraday RSD. Values for furosine were 6.4 to 10.1 for inter-days RSD and 3.1 to 9.1 for intraday RSD. Jalili and Ansari (2015) investigated 5-Hydroxymethyl furfural in food products and reported recovery values ranged from 84.4 to 105.8% [27]. In contrast to our results, these researchers did not detect HMF content in UHT milk.

others, but followed a descending trend (L, a, b) equations that use the square root of CIE (x, y, z) for L, a, b calculation, whereas the CIE (L*, a*, and b*) equations use the cubic root [30].

Table 1. Analytical parameters of proposed HPLC method for HMF and furosine detection*.

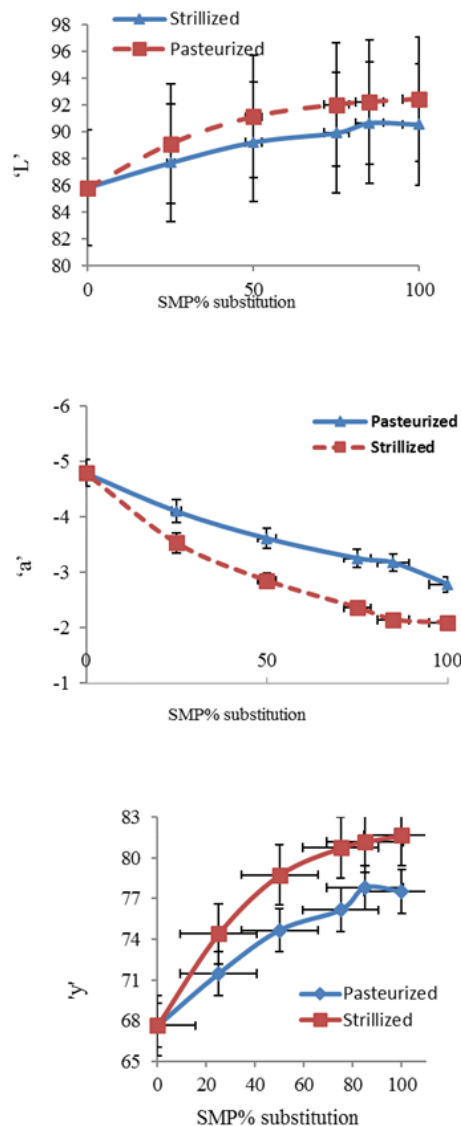| Parameter	HMF	furosine
Equation	y = 8.1667x + 4.5392	y = 6.3984x + 5.3233
Regression coefficient (R^2)	0.9924	0.988
LOD (ppm)	0.05	0.02
LOQ (ppm)	0.15	0.07

*RSD = relative standard deviation; LOD= Limit of detection; LOQ= Limit of quantitation.
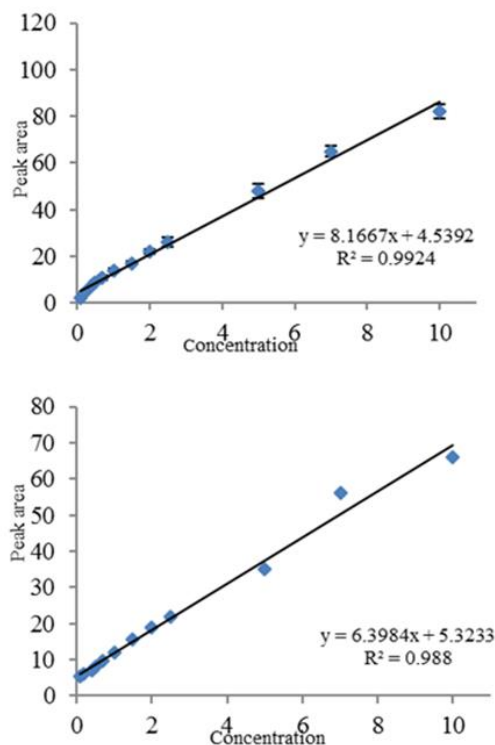
Figure 2. Calibration curve of furosine (a) and HMF (b).

Figure 3. Parameters 'L' (a), 'a' (b) and 'y' (c) of optical analysis in different treatments.

3.3. Optical determination

The International Commission on Illumination test (CIE test) is an acceptable reference test for qualification of the color of food and beverage. The CIELAB color scale is another uniform color scale recommended by the CIE in 1976 to improve on 1966 version of the Hunter L, a, b. It has been widely used in different industries. The primary difference in these two color systems is the Hunter. The curves related to the parameters 'L', 'a' and 'y' of treatments are presented in Figure 3. As can be seen, as expected, in parallel to an increase in milk solids non-fat, the amount of parameters 'L' and 'y' increased. On the other word, by increasing the SMP added to treatments, instead of their original solid non-fat, the 'a' value of the milk showed tendency to redness, but 'b' value to yellowish. In fact in heated milk, whiteness greenish-yellow tint of raw milk changed to reddish-yellow. Both 'a' and 'b' values have been used as indicators of the browning reactions [28]. The sterilized treatments had lower lightness due to the naturally subjecting to more severe heating during the processing compared to the pasteurized ones and occurring higher rates of non-enzymatic Maillard browning reactions [29]. The gap between the curves develops gradually as the amount of added SMP was increased. The curves of parameter 'a' were proportional to the

3.4. Determination of HMF and furosine.

Table 3 shows the amounts of HMF and furosine in different treatments. Both markers were not detected either in control pasteurized or in control sterilized samples indicating that the amount of the compounds produced in both mentioned processes has been less than the instrumental LOD in present study. Indeed, HMF and furosine are important indicators produced during the early stages of the Maillard reaction and are proportional to the severity of thermal treatments in foods. HMF can be also produced by caramelization [4, 5]. Furosine is formed from acid degradation

of lactulosyl-lysine during thermal treatment of milk. Production of furosine considerably increases as processing temperature increases for every 10°C, especially >95°C [31].

Considering Figure 4, there was a positive linear correlation between the increase of substituted SMP concentration and the concentration of both markers. According to Table 4, the levels of substituted MSNF by SMP in different commercial samples could be determined by investigating HMF and furosine contents with a good proportion. This could open a promising approach for the competent authorities in their surveillance programs. The greatest amounts of aforementioned indicators were observed in pasteurized and sterilized milk with 75% substitution by SMP. The resulted calibration curves by linear regression were linear with the correlation coefficient of 0.934 and 0.928 for HMF and furosine in pasteurized and sterilized samples, respectively (Table 4). Although contents of furosine higher than 2000 mg/l00 g of protein have been reported for powdered milk [11, 28], in most cases, the furosine ranged roughly from 100 to 400 mg/l00 g of protein [5, 14].

LOD in pasteurized and sterilized milk were 1.9 and 2.9, respectively. Bignardi et al. (2012) used a method for the qualitative and quantitative analysis of furosine in food products by capillary electrophoresis coupled to Tandem Mass Spectrometry and compared their results with HPLC method [24]. They reported LOD and LOQ values of 0.07 and 0.25mg/L respectively. The recovery was 77% at 4 ppm and 97% at 16 ppm; intra- and interday repeatability (RSD%) was equal to 4.6% and 14. [4, 5]. Morales et al. (2000) used an HPLC method for total HMF determination in industrial processed milk and reported 0.2 nmol/L as value [25]. Chen and Yan (2009) proposed a potentially rapid and reliable method to determine HMF in milk by capillary electrophoresis with diode array detection and reported 0. μg/mL as value for HMF [32]. Sabater et al. (2018) reported furosine in prebiotic-supplemented infant formula and samples without prebiotic ranging from 315-965 and 94-1226 mg/100 g protein, respectively [33].

Table 2. Recovery and precision Parameters of HMF and furosine for proposed determination method for concentrations of 0.5, 2.5 and 10 ppm.

Parameter	Analyt concentration (ppm)	HMF	furosine
Inter-days RSD% (n=5)	0.5	12.5	10.1
	2.5	9.3	7.8
	10	7.5	6.4
Intraday RSD% (n=3)	0.5	12.5	9.1
	2.5	5.9	4.1
	10	3.8	3.1
Recovery	0.5	73%	84%
	2.5	85%	91%
	10	89%	94%

Table 3. Analytical parameters for determination of HMF in real sample*.

Parameter	HMF in pasteurized sample	HMF in sterilized sample
Regression equation	y = 5.2311x + 41.187	y = 6.0628x + 130.93
Correlation of determination (R^2)	0.9347	0.9286
LOD (%)	1.9	2.9
LOQ (%)	6	10
Intra-day RSD%(n=5)	12	16
Inter-day RSD% (n=3)	4.5	5.9

*RSD = relative standard deviation; LOD= Limit of detection; LOQ= Limit of quantitation.
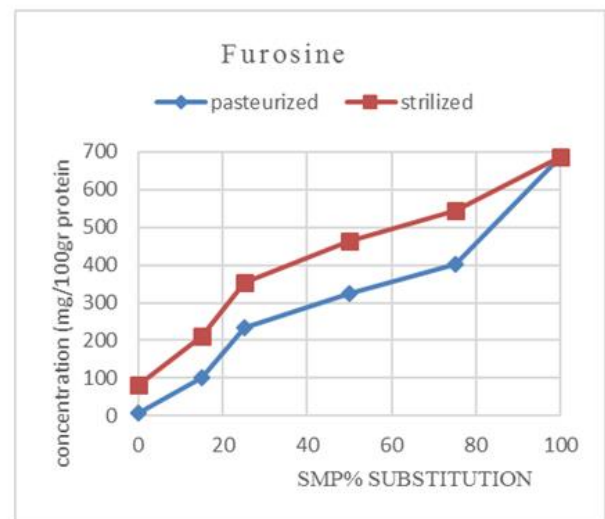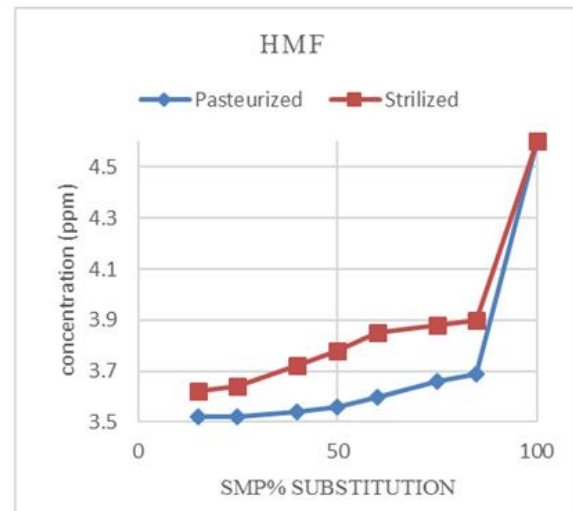
Figure 4. Correlation of SMP added and amounts of HMF and furosine in pasteurized and sterilized milks.

Table 4. HMF (μmol/L) and furosine contents (mg/100 g protein) of different treatments*.

Sample	HMF (μmol/L)	Furosine (mg/100 g protein)
Cp**	Nd	7.42[f]
P25	3.52[d]	233.33[d]
P50	3.56[c]	324.00[c]
P75	3.66[b]	402.34[b]
Cs	Nd	83.33[f]
S25	3.62[d]	353.32[d]
S50	3.78[c]	463.67[c]
S75	3.89[b]	544.66[b]
SMP	4.95[a]	685.21[a]

*Means in each column shown with different small English letters, are significantly (p<0.05) different.
**Cp = control pasteurized milk; Cs = control sterilized milk; P = pasteurized treatments; S = sterilized treatments. The numbers conjugated to 'P' or 'S' shows the percent of the original milk solid non-fat substituted by SMP.

3.5. Sensory evaluation

Table 5 shows the results of sensory evaluation of different treatments. As is evident, the most transparent samples were the controls (without SMP substitution), while the most turbid ones

were realized for those with 100% or 75% substitution. This could be ascribed to the higher amounts of Maillard browning reaction occurred in these treatments. The cooked flavor was realized in the treatments contained sterilized milk (the most, in control), while did not detect in other ones. This flavor note is formed mostly due to liberation of volatile sulfur compounds such as hydrogen sulfide from sulfur-containing amino acids in extent happens during the milk sterilization [34]. The characteristic powdery flavor emerges by drying of milk with spray drying method as a result of forming a complex of specific compounds during the Maillard reactions [35]. According to Table 5, the controls (pasteurized and sterilized milk) did not show any powdery flavor, and the greatest intensity of this feature was detected in the treatment with 100% substitution of MSNF by SMP.

Table 5. Sensory evaluation of treatments*.

Sample	Cooked flavor	Powdery flavor	Color
Cp**	1^e	1^e	2.10^a
P$_{25}$	1^e	2.32^d	1.92^b
P$_{50}$	1^e	3.40^c	2.03^{ab}
P$_{75}$	1^e	4.12^b	2.07^a
Cs	4.21^a	1^e	2.12^a
S$_{25}$	3.52^b	2.43^d	1.96^b
S$_{50}$	2.86^c	3.52^c	2.03^{ab}
S$_{75}$	1.95^d	4.18^b	2.05^a
SMP	1^e	4.96^a	2.10^a

*Means in each column shown with different small English letters, are significantly ($p<0.05$) different.
**Cp = control pasteurized milk; Cs = control sterilized milk; P = pasteurized treatments; S = sterilized treatments. The numbers conjugated to 'P' or 'S' shows the percent of the original milk solid non-fat substituted by SMP.

4. CONCLUSIONS

In the current study, detection of original pasteurized and sterilized cow's milk from those with partial reconstitution (partial substitution of original MSNF by SMP) was successfully carried out by determination of two markers of Maillard reaction including furosine and HMF. The method validation evaluation was performed. Overall, this study could be hopefully used by the competent authorities to ensure regulation correspondence. There was a good correlation between the two major metabolites and the amount of added SMP as well as the sensory characteristics of the final product, which could be led to find mentioned milk adulteration.

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