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Research Article

RP-HPLC determination of Furosine in fermented milk of different brands retailed in China

Abstract

Furosine (ε-N-2-furoylmethyl-L-lysine) is the most specific suitable indicator of the severity of heat treatment and storage conditions of dairy products. The objective was to determine furosine content in fermented milks by ion-pair reversed-phase high-performance liquid chromatography (RP-HPLC). In this study, furosine quantified in 27 different brands of fermented milk retailed in China. The concentrations of furosine in the fermented milk ranged from 25.40 ± 5.2 to 1661.05 ± 89.9 mg/100 g of protein. About 26 % of the samples in the present study had shown remarkably a very high furosine mean values. Furosine may, therefore, be a good indicator of the degree of Maillard reaction induced damage during the processing of fermented milks.

Abbreviations

RP-HPLC: Reversed-Phase High-Performance Liquid Chromatography; UV: Ultraviolet; UHT: Ultra-High Temperature; ESL: Extended Shelf-Life; IEC: Ion-Exchange Chromatography.

Introduction

Heat treatment of milk is the most widely used processing technology in the dairy industry. It results in many chemical and biochemical changes in milk, the extent of which depends on the temperature-time combinations, the heating method utilized, and milk pre-treatment conditions [1-3]. The principal categories of treatment methods used for milk for direct consumption and *those used for* specific dairy products are in-container sterilization, UHT, ESL processing, and pasteurization [4,5].

The quality of milk is affected by heating treatments as a consequence of interactions between the amino acid lateral groups, restructuring of –SH and S–S– groups, insolubilizations of whey protein, interactions between k-casein and β-lactoglobulin, hydrolysis of lipids, degradation of lactose to organic acids, destruction of some vitamins and enzymes, and disturbance of calcium/phosphorous equilibrium [6,7]. Effects during the heating of milk; Firstly, the effect on milk components of particular interest for keeping quality and nutritional properties of milk. The second type of effects related to cooked flavor and loss of nutritional value due to the formation of new substances formed by Maillard reaction, which continues during the storage of heated milk [8].

Furosine (ε-N-2-Furoylmethyl-L-lysine) is an amino acid formed during acid hydrolysis of the main stable Amadori compound (ε-deoxy-fructosyllysine), and used to measure the initial steps of the Maillard reaction [9,10]. It is considered a useful indicator of the severity of heat treatment, Maillard reaction related to the identification of limiting values of thermal damage, and poor storage conditions of the finished products [7,11-14]. In addition, furosine is a useful chemical parameter for evaluating the quality and possible detection of food frauds, especially in milk and milk-based products, such as yogurt and milk powders [15-17].

Determinations of furosine could be carried out by gas chromatography, IEC and HPLC. Now, HPLC is the technique of choice since it provides the best detection limit and is not time-consuming [7,18,19]. More recently, [12] proposed an ion-pair reversed-phase HPLC method for the determination of furosine in acid-hydrolyzed dairy products. At present, no published data available on the furosine contents of fermented milk in China. This study was, therefore, conducted to determine furosine content in fermented milk retailed in China by ion-pair reversed-phase high-performance liquid chromatography.

Materials and Methods

Samples

Twenty-seven different commercial brands of fermented

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milk samples retailed in China were purchased. Their expiration dates ranged from July 2018 to March 2019. Samples were kept frozen after aliquoted until further analyses. All analysis was done in triplicate unless otherwise indicated.

Reagents and standards

All reagents used for sample preparation were of analytical grade, and solvents for chromatographic analysis were of HPLC grade. Throughout all experiment, ultrapure water was used (SG Ultra Clear UV system; Siemens Water 95 Technologies, Warrendale, PA, USA). All reagents required for experimental analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA) including Acetonitrile (ACN, 100%), Hydrochloric acid (37%), Methanol (99.8%), Formic acid (FA, 98%), Trifluoroacetic acid (TFA, 99%). Furosine standard (furosine dihydrochloride; 99.1%) purchased from Iris Biotech Laboratories GmbH (Marktredwitz, Germany).

RP-HPLC conditions

Column type: C18; Length: 250 mm; I.D. 4.6 mm (Alltech Furosine-dedicated); Column temperature: 30°C; U.V. detection: 280 nm; Flow rate: 0.6 mL/min; Injection volume: 10µL; Runtime: 30min; Solvent A: 0.1% Trifluoroacetic acid solution; Solvent B: Methanol; HPLC apparatus: Waters Model 2996. The elution gradient is given in Table 1.

Table 1: Elution gradient.

Time (min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0.00	0.6	95	5
16.00	0.6	86.8	13.2
16.50	0.6	5	95
25.00	0.6	95	5
30.00	0.6	95	5

RP-HPLC analysis of furosine with UV detection

For determination of furosine content, samples were prepared according to [12,20-22] with very few modifications in sample preparation. Briefly, all samples were pre-warmed in a water bath set at 40 °C for 15 min, to get a homogeneous solution. 2mL of fermented milk samples were mixed with 6mL of 10.6 N hydrochloric acid and exposed to nitrogen for 2 min, expelling oxygen. Subsequently, tubes were closed tightly and heated at 110 °C for 23 h for acid hydrolysis in screw-cap Pyrex® tubes. The hydrolysate was brought to room temperature and filtered through a Whatman 5951/2 filter paper. The filtrate was further processed using reversed-phase solid-phase extraction (SPE) Sep-Pak C18 cartridge (Waters Corporation, Milford, MA 01757, USA). 0.5 mL of the filtrate was eluted with 3 mL of 3 N hydrochloric acid and evaporated under vacuum. 1mL of the dried samples were reconstituted with 3.5 mL of a mixture of 5% v/v acetonitrile/0.2% v/v formic acid and filtered through a 0.22-µm filter (Millex PVDF; Millipore) before HPLC injection. Eighty microlitres of each sample injected onto a SUPELCO C18 column (5µm, 250 × 4.6 mm, I.D.) (Waters Corporation, MA, USA). Calibration of the chromatographic system for furosine (FUR) determination was by the external standard method. A standard stock solution containing 200 µg FUR mL^{-1} was used to prepare the standard working solution. Calibration curves (0, 2, 4, 8, 16 32, 64 and 128 µg FUR mL^{-1}) were carried out by plotting absorbance, with the peak area as the ordinate and the furosine concentration is the abscissa. The regression equation for the standard curve: $y = 35.414x + 9.2669$; $R^2 = 0.9997$. The obtained results presented as mg/100 g of protein (Table 2). The furosine value usually expressed as mg/100 g of protein [23].

Determination of total protein content

The total nitrogen content determined according to [24] Kjeldahl method and required for the final furosine calculation of samples. A factor of 6.38 was applied to convert nitrogen values to protein.

Statistical analysis

All experimental analysis was carried out in triplicate, and the results are expressed as means ± standard deviation (SD). Calculations of mean values, standard deviations, and correlation coefficient (R^2) accomplished with Microsoft Excel 2016. The obtained data were analyzed with one-way analysis of the variance (ANOVA)and post-tested by Tukey test using GraphPad.Prism.7.00 software and differences were considered significant at $P < 0.05$, with a 95% confidence interval.

Results and Discussion

The typical HPLC chromatograms of furosine standard and fermented milk are presented in figure 1 and compared to the data reported by different authors [7,12,25], a shorter retention time observed in our investigation. This difference observed in the present study may arise from a variety of factors, including the type of column used and chromatographic conditions elaborated in the method.

Table 2 shows the results of concentrations of furosine in all investigated samples. The concentrations of furosine in the fermented milk of different brands ranged from 25.40 ± 5.2 to 1661.05 ± 89.9 mg/100 g of protein. Nearly 26 % of the samples in the present study had shown significantly higher furosine mean values than reported by other authors [26]. Remarkably, sample CF, JC, and XM showed a very high mean furosine content. The highest concentration of furosine obtained could be because of the addition of milk powder into fermented milk [27,28]. According to a report of the previous studies [28-32],

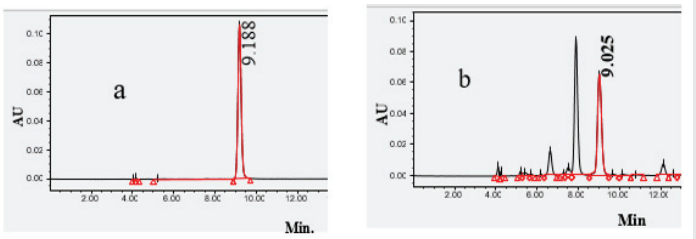

Figure 1: Typical HPLC chromatograms: (a) Furosine standard (9.188 min.) and (b) Fermented milk (9.025 min).

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Table 2: Data from the label information and quantified furosine contents (mg/100 g of protein) in fermented milk retailed in China (n = 27). Results are presented in order of decreasing furosine contents.

(Code)[1]	(Brand)[2]	Protein content (%)		Furosine content (mg/100 g of protein)[5]
		(Label)[3]	(Analyzed)[4]	
109	CF	2.5	3.0	1661.1±89.9a
3	JC	2.8	3.0	1279.6±49.7[b]
51	XM	2.6	2.8	986.2±28.6c
19	QF	3.2	3.6	445.5±84.5[d]
13	JG	2.5	2.7	374.7±24.1[d,e]
78	XI	3.0	3.3	334.3±27.5[e]
77	SM	3.0	3.0	321.3±39.1[e]
47	NH	6.2	6.2	211.4±3.1[f]
46	WS	3.0	2.6	177.2±4.7[f,g]
134	MN	3.7	3.7	143.7±2.3[f,g,h]
125	GC	3.3	3.8	135.1±28.1[f,g,h]
11	EY	3.0	2.8	130.2±15.9[f,g,h]
68	GF	3.3	3.3	125.4±21.7[f,g,h]
17	YM	3.0	3.3	117.7±7.2[f,g,h,i]
75	SC	3.1	3.3	112.5±1.3[g,h,i]
29	KL	3.0	3.2	110.7±13.1[g,h,i]
140	WF	3.1	3.1	104.8±8.2[g,h,i]
115	SY	3.1	3.5	101.6±8.2[g,h,i]
88	SN	3.1	2.9	95.7±3.6[g,h,i]
21	SS	3.1	2.7	81.6±3.3[h,i]
67	FU	4.6	4.7	70.8±6.8[h,i]
81	LC	7.2	7.4	60.9±14.6[h,i]
92	UG	4.8	4.0	60.1±7.8[h,i]
6	JS	3.8	3.6	50.9±0.5[h,i]
90	LJ	7.2	7.2	48.2±0.6[h,i]
34	YF	3.1	3.0	37.7±2.1[hi]
37	JO	2.8	3.2	25.4±5.2[i]

[1] Sample identification; [2] Identifies the manufacturer; [3]Obtained from the label information [4] N × 6.38; [5] Mean±SD

[a-i]Means values with different superscripts within the same column shows significantly different from each other at (p<0.05).

the amount of furosine related to the hydrolysis of the amino acid, the temperature of the production process and duration of exposure to the temperature, as well as storage temperature of the finished product. Likewise, milk proteins which are rich in lysine residues are prone to Maillard reaction, and consequently, the Amadori product rapidly formed under slight heating conditions [33].

The concentration of furosine on dairy products is used as an indicator for assessing the effects of the thermal treatments applied to milk and, also serves as a chemical parameter useful for nutritional evaluation of foods regarding protein and lysine contents, as an estimate of protein quality [33]. The intermediate and end-products of Maillard reactions, i.e., furosine, which is produced when milk and milk products are heated, exert toxicological consequences such as potent neuro-toxicants to human. Furthermore, High doses of furosine cause adverse effects on health by inducing cell apoptosis and activation of inflammatory [34,35].

On the other hand, about 74% of the samples had shown a furosine level below 300 mg/100 g of protein. These results are in disagreement with other authors [36] who found furosine content of 316.47 ± 0.17 mg/100 g of protein in the fermented milk. The lower furosine content obtained in this study may indicate features of the production lines like heating under reduced pressure and falling film evaporation technology [37].

A statistically significant difference observed ($p < 0.05$) in furosine concentrations between the different brands of fermented milk, as shown in table 2. The significant variability found between samples is probably due to the heat-treatment intensity given to fermented milk and fermented milk-making conditions [26]. It is difficult to compare the furosine content of the present study with those of previous work because of very few published data found concerning the furosine content in fermented milk.

Acceptable levels of furosine 3 to 5.5, 4 to 7, and 220 to 372 mg/100 g protein for raw milk, pasteurized milk, and sterilized milk, respectively [11,38,39]. However, to the best of authors knowledge, there was no specification set for the maximum permissible level of furosine in fermented milk in China.

Conclusions

The present study investigated the quantitative analysis of furosine content in fermented milk retailed in China. The method was highly sensitive and rapid for the determination of furosine in dairy products such as yogurt and other fermented milk. According to the obtained results, careful attention to the quality of fermented milk required because of the abnormal increment of furosine concentrations observed and detected differences. This study also suggests an urgent need for the maximum legal limits of furosine in fermented milk.

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