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Original article

RP-HPLC analysis of furosine and acid-soluble β-lactoglobulin to assess the heat load of extended shelf life milk samples in Austria

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Abstract – Recent trends to extend the shelf life of pasteurized milk, without the negative flavour normally associated with ultra-high-temperature (UHT) milk, have resulted in the development of extended shelf life (ESL) milk. Acid-soluble β-lactoglobulin (β-Lg) and furosine contents were chosen as relevant indicators for heat load of ESL milk products. RP-HPLC methods were developed using the same column (Symmetry 300™, Waters), which enabled the separation of whey proteins within 22 min; furosine was analysed using ion-pair RP-HPLC within 8 min. Electrophoresis was used as a high-throughput and cost-effective screening method to assess the impact of thermal processes on milk and to distinguish different categories of heat-treated milk samples. Liquid milk samples (n = 128; including 7 raw, 33 pasteurized, 71 ESL, and 17 UHT milk samples) were obtained from retail outlets in Austria and analysed. Only 45% of the analysed samples designated as ESL milk showed furosine contents < 40 mg·100 g^{-1} protein as well as acid-soluble β-Lg contents > 1800 mg·L^{-1} milk, which had been discussed as threshold levels for ESL milk. A further 55% of the analysed ESL milk samples had low acid-soluble β-Lg (< 500 mg·L^{-1}) and high furosine contents (> 40 mg·100 g^{-1} protein), levels comparable to the excessive heat load of UHT milk. Thus, there is an urgent need for an EU regulation to define legal limits for the tolerable heat load of ESL milk as soon as possible.

extended shelf life milk / β-lactoglobulin / furosine / RP-HPLC / electrophoresis

摘要 – RP-HPLC 法测定糠氨酸和酸溶性 β-乳球蛋白及推测澳大利亚 ESL 乳的热负荷。近年来，长保质期的巴氏杀菌乳 (ESL) 由于其避免了 UHT 乳风味差的优点而得到了广泛的推广。本文采用反相-高效液相色谱法测定乳中糠氨酸和 β-乳球蛋白含量，以此推测 ESL 乳的热负荷。HPLC 法可以在 22 min 内有效地分离乳清蛋白，而采用反相离子对色谱法可以在 8 min 内分析糠氨酸。电泳法作为一种高通量和有效的检测方法可以评价热处理对乳的影响和区分不同类型热处理的乳样品。取 128 个零售的乳样品 (其中 3 个生鲜乳、33 个巴氏杀菌乳、71 个ESL 乳、17 个 UHT 乳)，根据分析其糠氨酸和 β-乳球蛋白含量来推测热负荷。在 ESL 乳中，45% 样品中每 100 克乳蛋白的糠氨酸含量低于 40 mg，每升乳中β-Lg 含量高于 1800 mg，这两个值低于 ESL 奶热负荷极限值。而另外 55% 样品则有较高的糠氨酸含量 (> 40 mg·100 g^{-1}蛋白) 和较低的 β-Lg 含量 (< 500 mg·L^{-1})，这一数值与 UHT 乳的热负荷相当。因此，有必要建议欧盟尽快制定法规来定义 ESL 乳的热负荷极限。

长保质期乳 (ESL) / β-乳球蛋白 / 糠氨酸 / 反相-高效液相色谱 / 电泳

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Résumé – Analyse de la furosine et de la β-lactoglobuline soluble à pH acide par RP-HPLC pour évaluer la charge thermique de laits de longue conservation en Autriche. La tendance actuelle visant à allonger la durée de conservation du lait pasteurisé, sans avoir les défauts de flaveur habituellement associés au lait UHT, a conduit au développement du lait de longue conservation ESL. Les concentrations en β-lactoglobuline soluble à pH acide (β-Lg) et en furosine ont été choisies comme indicateurs pertinents de la charge thermique des produits laitiers ESL. Des méthodes RP-HPLC ont été développées avec la même colonne (Symmetry 300™, Waters), qui a permis de séparer les protéines sériques en 22 min ; la furosine était analysée par RP-HPLC en paire d'ions en 8 min. L'électrophorèse a été utilisée comme méthode économique de criblage à haut débit pour évaluer l'impact des traitements thermiques sur le lait et pour distinguer différentes catégories d'échantillons de lait traité thermiquement. Les échantillons de lait liquide (n = 128 ; comprenant 7 échantillons de lait cru, 33 pasteurisé, 71 ESL et 17 UHT) ont été prélevés dans des points de vente en Autriche et analysés pour leur teneur en β-lactoglobuline et furosine. Seulement 45 % des échantillons analysés désignés comme lait ESL présentaient des teneurs en furosine inférieures à 40 mg·100 g^{-1} de protéine et des teneurs en β-lactoglobuline soluble à pH acide supérieures à 1800 mg·L^{-1}, valeurs qui ont été discutées pour être retenues comme niveaux seuils pour le lait ESL. D'autre part, 55 % des échantillons de lait ESL analysés avaient des teneurs basses en β-lactoglobuline soluble à pH acide (< 500 mg·L^{-1}) et élevées en furosine (> 40 mg·100 g^{-1} de protéine), ces niveaux étant comparables à ceux obtenus pour du lait UHT à charge thermique excessive. En conséquence, il existe un besoin urgent de réglementation au niveau européen pour définir au plus vite les limites obligatoires en matière de charge thermique tolérable pour le lait de longue conservation ESL.

lait de longue conservation / β-lactoglobuline / furosine / RP-HPLC / électrophorèse

1. INTRODUCTION

The relatively short shelf life of pasteurized milk has resulted in the development of ultra-high-temperature (UHT) treated milk for ambient distribution, which has gained widespread acceptance in many countries. In other areas, however, consumers have not accepted UHT milk because of the perceived "cooked" taste of the product. Consequently, the need for extending the shelf life of pasteurized milk, without the negative flavour change normally associated with UHT, has resulted in the development of milk products with taste similar to pasteurized milk, but with some of the obvious benefits of longer keeping ability of the product. The currently used methods to produce extended shelf life (ESL) milk are microfiltration, direct heat treatment such as injection or infusion (e.g. 127 °C for 2–3 s), or in many cases also indirect heat treatment (e.g. 125 °C for 2 s) [15, 18, 24, 25]. However, heating causes a significant loss of organoleptic and nutritional quality (e.g. vitamin destruction, precipitation of calcium phosphate, denaturation of whey proteins, and Maillard reaction) [2, 8, 10, 11, 22]. Furthermore, an undesirable precipitation of denatured proteins and minerals can be formed on the walls of heat exchangers [15, 18, 25].

To quantify the impact of thermal processes on milk, time temperature integrators (TTIs) can be used for heat-load evaluation. Several milk compounds have been suggested as potential TTIs for the assessment of heat treatment of milk (e.g. the enzymes alkaline phosphatase and lactoperoxidase; the whey protein β-lactoglobulin β-Lg; hydroxymethylfurfural, HMF; lactulose; and furosine) [3–6, 20, 22]. Type I reactions include the denaturation, degradation, and inactivation of heat-labile components (mainly whey proteins, enzymes, and vitamins) – these indicators are most suitable tools for the evaluation of low-heat treatments, whereas type II reactions include

the formation of substances that are (almost) not present in unprocessed milk (e.g. lactulose, HMF, and furosine) – these indicators are more effective for the assessment of processes involving high temperatures [3, 4, 22].

The quantitative determination of acid-soluble β-Lg has been proposed to distinguish between different categories of heat-treated milk. A minimum content of 2600 mg·L^{-1} for pasteurized milk, of 2000 mg·L^{-1} for high-pasteurized milk, and of 50 mg·L^{-1} for UHT milk is within the limits proposed by the International Dairy Federation. In addition, furosine has also been proposed as a useful index for heat-induced changes in milk products. A furosine content of 8 mg·100 g^{-1} protein has been suggested as upper limit for pasteurization, of 20 mg·100 g^{-1} protein for high pasteurization, and of 250 mg·100 g^{-1} protein for UHT processing [3, 4, 6]. At present, for lack of obligatory limits regarding the heat load of ESL milk in Europe, some rather general recommendations are circulating (β-Lg > 1800 mg·L^{-1}; furosine < 12 mg·100 g^{-1} protein; and lactulose < 30 mg·L^{-1}) [9, 15], which are taken seriously by individual dairy companies, but are neglected or completely ignored by all the others without any consequences by the control authorities. Moreover, as this "new" category of liquid milk has to be cooled also at < 6 °C, pasteurized milk is currently being displaced in all supermarkets by ESL milk products. However, because of the partly changed nutritional and sensory quality of these products, this recent development towards an increasing consumption of milk "ESL" may have minor consequences for human nutrition in future.

The objective of this study was to improve RP-HPLC methods for the analysis of furosine and acid-soluble β-Lg in milk using the same column and to determine the heat load of different categories of heat-treated liquid milk samples taken from retail outlets in Austria. Moreover, electrophoresis of acid-soluble whey proteins was used to assess the impact of thermal processes on market milk.

2. MATERIALS AND METHODS

2.1. Milk samples

Commercial liquid milk samples (n = 128) from different categories of heat treatment produced by different dairy companies were taken from retail outlets in Austria (see Tab. I). Samples of raw milk (n = 7), pasteurized milk (n = 33), ESL milk labelled with "länger frisch" (i.e. longer fresh) (n = 71), and UHT milk (n = 17) were aliquoted and kept frozen until consecutive chromatographic and electrophoretic analyses.

2.2. RP-HPLC analysis of acid-soluble β-lactoglobulin

Quantitative determination of acid-soluble β-lactoglobulin in liquid milk samples was performed using RP-HPLC following the IDF standard [14] with some modifications. Briefly, caseins and denatured whey proteins were precipitated at pH 4.6 by the dropwise addition of 2 mol·L^{-1} HCl. Acid whey containing the acid-soluble whey proteins soluble at pH 4.6 was separated by centrifugation and diluted (1:10 or 1:5 in the case of UHT milk) with sodium phosphate buffer solution (100 mmol·L^{-1}, pH 6.7). Samples were filtered through 0.20 µm Minisart RC 4 filters (Sartorius, Goettingen, Germany).

RP-HPLC was performed on a Waters chromatography system using a model 600E multisolvent delivery system, a Rheodyne 7725i injector, guard column (Sentry Guard, Symmetry™ C_{18}, 3.5 µm, 2.1 × 10 mm), and a Symmetry™ 300 C_{18} column (3.5 µm, 2.1 × 150 mm) (Waters Corporation, Milford, MA, USA).

Table I. Acid-soluble β-Lg ($mg \cdot L^{-1}$) and furosine ($mg \cdot 100\ g^{-1}$ protein) contents in different categories of heat-treated milk samples taken from retail outlets in Austria ($n = 128$). Results are given in order of decreasing β-Lg contents of milk samples within each category.

Code	Expiry date	Brand[1]	Fat[2] (%)	Protein (%)	Furosine ($mg \cdot 100\ g^{-1}$ protein)	β-Lg ($mg \cdot L^{-1}$)
			Raw milk samples ($n = 7$)			
123	11.01.2009	**A**	Nat.[3]	3.41	5.9	5215
125	11.01.2009	**B**	Nat.[3]	4.43	8.7	5210
47	04.04.2008	**C**	Nat.[3]	3.61	10.0	3752
101	11.05.2008	**D**	3.6	2.93	7.6	3706
59	13.04.2008	**D**	3.6	3.13	7.0	3620
100	02.05.2008	**C**	Nat.[3]	3.21	8.4	3367
62	11.04.2008	**C**	Nat.[3]	3.24	8.5	3261
			Pasteurized milk samples ($n = 33$)			
9	11.12.2007	**E**	3.5	3.71	10.2	3799
80	22.04.2008	**F**	1.8	3.29	10.3	3635
56	11.12.2007	**F**	3.5	3.57	10.0	3606
45	09.04.2008	**E**	3.5	3.18	9.6	3443
129	19.12.2008	**G**	3.6	3.18	9.7	3429
85	28.04.2008	**H**	3.6	3.30	8.6	3418
131	19.01.2009	**G**	3.6	3.21	9.5	3417
106	13.05.2008	**I**	1.5	3.09	9.5	3358
84.3	29.04.2008	**J**	3.5	3.28	10.0	3357
84.1	27.04.2008	**F**	3.5	3.34	8.4	3355
107	13.05.2008	**I**	3.8	3.03	10.3	3332
105	12.05.2008	**I**	1.5	3.14	9.2	3329
99	09.05.2008	**F**	3.5	3.28	9.7	3325
93	06.05.2008	**K**	3.5	3.33	13.3	3305
58	12.04.2008	**L**	3.5	3.20	11.3	3301
108	16.05.2008	**M**	3.5	3.31	9.2	3280
69	17.04.2008	**N**	3.5	3.17	9.6	3108
120	06.06.2008	**N**	3.5	3.25	8.3	3082
113	12.05.2008	**N**	3.5	3.25	8.7	3061
114	22.05.2008	**N**	3.5	3.24	10.4	3052
35	31.03.2008	**O**	3.5	3.22	9.7	3049
130	11.01.2009	**G**	3.6	3.18	9.9	3045
118	31.05.2008	**F**	3.5	3.21	8.1	3041
55	10.12.2007	**G**	3.8	3.16	12.3	3025
104	09.05.2008	**O**	3.5	3.04	8.8	2989
54	10.12.2007	**G**	1.6	3.40	9.8	2951
111	16.05.2008	**G**	1.6	3.32	10.8	2937
78	21.04.2008	**P**	3.5	3.39	11.0	2858
14	29.02.2008	**G**	4.5	3.06	10.9	2823
116	23.05.2008	**P**	3.5	3.25	10.0	2748
115	23.05.2008	**G**	Nat.[3]	3.14	8.8	2739
121	07.06.2008	**P**	3.5	3.38	10.2	2697
33	31.03.2008	**J**	3.5	3.32	11.4	2528
			ESL milk samples ($n = 71$)			
88	06.05.2008	**Q**	0.5	3.30	11.7	3679
95	19.05.2008	**R**	3.5	3.42	11.0	3523
94	09.05.2008	**R**	3.5	3.36	12.0	3501

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Table I. Continued.

Code	Expiry date	Brand[1]	Fat[2] (%)	Protein (%)	Furosine (mg·100 g^{-1} protein)	β-Lg (mg·L^{-1})
87	10.05.2008	**Q**	3.5	3.39	16.3	3433
71	27.04.2008	**M**	3.5	3.31	11.3	3371
34	16.04.2008	**J**	1.5	3.58	13.3	3246
92	14.08.2008	**R**	3.5	3.42	11.5	3223
96	09.05.2008	**R**	3.5	3.32	13.6	3125
60	23.04.2008	**M**	3.5	3.09	12.1	3088
21	20.12.2007	**K**	1.5	3.83	13.3	2866
126	25.01.2009	**S**	3.5	3.48	12.8	2858
127	29.01.2009	**T**	3.5	3.43	11.6	2792
72	01.05.2008	**M**	1.5	3.44	13.7	2663
98	21.06.2008	**U**[4]	0.7	3.86	37.1	2653
23	03.12.2007	**M**	1.5	3.54	15.2	2541
79	07.05.2008	**M**	3.5	3.37	15.3	2533
7.2	10.12.2007	**T**	3.5	3.16	13.8	2475
52	25.04.2008	**V**	3.5	3.18	11.3	2431
84.2	16.05.2008	**J**	1.5	3.25	10.2	2406
119	06.06.2008	**S**	3.5	3.18	14.0	2404
6	30.11.2007	**M**	3.5	3.24	15.9	2402
109	03.06.2008	**J**	1.5	3.47	11.8	2390
50	10.05.2008	**U**[4]	0.7	3.90	39.4	2311
103	27.05.2008	**P**	3.5	3.33	13.1	2270
1	14.12.2007	**W**	3.5	3.45	15.6	2259
26	29.11.2007	**U**[4]	0.7	4.02	29.3	2245
102	21.05.2008	**X**	3.5	3.10	15.5	2180
17	26.12.2007	**Y**[4]	3.8	3.49	26.9	2025
81	11.06.2008	**Y**[4]	3.8	3.17	17.3	1946
49	08.04.2008	**S**	3.5	3.32	24.5	1839
18	11.03.2008	**S**	3.5	3.32	23.6	1827
42	07.05.2008	**Y**[4]	3.8	3.26	22.5	1801
86	02.05.2008	**Z**	3.5	3.30	40.2	430
12	15.03.2008	**G**	1.0	3.14	50.5	401
20	17.03.2008	**F**[5]	1.5	3.28	123.9	387
82	10.05.2008	**F**[5]	1.5	3.35	135.2	386
28	16.03.2008	**F**	3.5	3.30	41.4	379
61	26.04.2008	**F**[5]	1.5	3.45	140.3	374
73	04.05.2008	**F**	3.5	3.13	40.8	336
16	09.12.2007	**F**	3.5	3.65	40.6	333
124	02.02.2009	**M**	1.5	3.58	81.8	330
13	16.03.2008	**G**	3.5	3.21	49.3	328
44	25.04.2008	**G**	1.0	3.28	50.0	322
2	05.12.2007	**G**	1.0	3.39	47.6	306
122	18.01.2009	**G**	3.6	3.19	49.8	300
110	07.06.2008	**G**	1.0	3.12	52.7	298
112	02.06.2008	**G**	3.6	3.00	53.3	291
134	08.02.2009	**G**	3.6	3.19	45.4	278
31	13.12.2007	**R**	0.1	3.8	74.7	260
7.1	13.12.2007	**V**	3.5	3.31	75.9	245
51	25.04.2008	**R**	0.1	3.67	82.4	240
3	20.02.2008	**M**	1.5	3.74	80.2	239
22	09.12.2007	**M**	3.5	3.39	72.0	234

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Table I. Continued.

Code	Expiry date	Brand[1]	Fat[2] (%)	Protein (%)	Furosine (mg·100 g^{-1} protein)	β-Lg (mg·L^{-1})
83	07.05.2008	**M**	1.8	3.51	100.1	231
137	25.01.2009	**G**	1.0	3.27	50.3	230
15	13.12.2008	**J**	1.5	3.59	77.9	229
132	09.01.2009	**G**	3.6	3.16	48.3	226
74	03.05.2008	**P**	3.5	3.17	94.2	226
135	08.01.2009	**G**	1.0	2.98	46.9	217
25	26.03.2008	**M**	1.8	3.81	62.2	213
24	18.03.2008	**M**	1.8	3.66	46.2	212
57	23.04.2008	**X**	3.5	3.13	82.0	212
11	13.03.2008	**V**	3.5	3.02	94.1	211
19	17.03.2008	**M**	0.9	3.45	95.1	206
4	04.12.2007	**M**	1.8	3.75	82.5	205
77	08.05.2008	**M**	1.8	3.58	74.2	204
136	25.01.2009	**G**	1.0	3.26	52.4	192
133	26.01.2009	**G**	3.6	3.21	40.1	186
76	06.05.2008	**M**	0.9	3.42	92.5	184
91	20.06.2008	**AA**[4]	0.1	3.79	262.2	160
10	02.04.2008	**AA**[4]	0.1	4.15	225.5	140
			UHT milk samples (n = 17)			
41	18.04.2008	**M**[5]	1.8	3.50	284.7	403
75	14.07.2008	**R**	3.5	3.39	159.2	344
39	23.03.2008	**BB**	0.1	3.64	93.1	256
36	28.05.2008	**R**	0.5	3.48	160.3	244
48	15.03.2008	**E**	0.5	3.66	151.6	232
68	10.07.2008	**F**	0.5	3.30	165.5	221
43	14.06.2008	**CC**[5]	1.5	3.44	352.4	220
40	09.03.2008	**R**	3.5	3.56	161.3	216
66	06.06.2008	**BB**	3.8	3.26	109.1	214
38	17.05.2008	**DD**	1.5	3.62	138.9	213
117	15.07.2008	**R**	0.5	3.55	168.1	196
90	14.08.2008	**EE**	0.5	3.53	137.8	183
89	28.07.2008	**EE**	3.5	3.39	181.5	172
8	02.03.2008	**R**	0.5	3.21	162.8	170
37	01.04.2008	**AA**[5]	1.5	3.32	207.0	166
97	20.08.2008	**FF**[5]	3.5	3.34	471.9	159
67	22.07.2008	**FF**[5]	3.5	3.20	485.0	158

[1] Bold values are anonymous designation of products.
[2] Fat content according to labelling.
[3] Natural fat content (min. 3.5%).
[4] Protein-enriched milk sample.
[5] Lactose-free milk sample.

Column eluates were monitored at 205 nm using a Waters 2489 UV/VIS Detector interfaced with a PC running Waters Millennium[32] chromatography manager. After flushing the column for 20 min with solvent B [0.1% (v/v) trifluoroacetic acid (TFA) in acetonitrile], the initial conditions were set until a stable baseline was observed (~ 30 min): 64% solvent A [0.1% (v/v) TFA in ultra-high quality (UHQ) water] and 36% solvent B. Samples (10 µL) were applied in duplicate onto the column and eluted at 40 °C at a flow rate

of 0.35 mL·min^{-1} using the following optimized gradient: from 36% to 50% solvent B linearly over 14 min, increasing to 100% B within 0.5 min, and finally holding at 100% B for 3.5 min. The column was then subsequently returned to the initial conditions within 1 min and equilibrated for 15 min before the next sample injection. β-lactoglobulin with a purity of ~ 90% (Sigma Chemical Co., St. Louis, MO, USA) was used as standard for calibration. Calibration curve (0.2, 0.4, 0.8, 1.6, 2.4, and 3.2 μg·10 μL^{-1} injection volume) was obtained by plotting peak area versus microgram of β-Lg. Concentrations of β-Lg were displayed as microgram, which were subsequently converted to the results given in mg·L^{-1} milk (Tab. I).

2.3. RP-HPLC analysis of furosine

Although an IDF standard exists also for the determination of furosine content in milk [13], an ion-pair RP-HPLC method was developed to analyse furosine [ε-*N*-(2-furoylmethyl)-L-lysine] using the same column as for β-Lg. Sample preparation including acid hydrolysis and cleaning of hydrolysates using solid-phase extraction (SPE) prior to chromatographic separation was performed according to the IDF standard [13] with some modifications. Briefly, 2 mL milk sample (UHT milk was diluted 1:4) was hydrolysed in the presence of 6 mL of 10.6 mol·L^{-1} HCl for 23 h at 110 °C in screw-capped Pyrex tubes (after bubbling with nitrogen for 2 min). The hydrolysate was filtered (Schleicher & Schuell 595½) and applied to SPE to minimize contamination: 0.5 mL of hydrolysate was added to a pre-wetted (5 mL methanol and 10 mL water) Sep-Pak® Vac 3 cc (500 mg) C_{18} cartridge (Waters); the eluted liquid was discarded, and furosine was then eluted with 3 mL of 3 mol·L^{-1} HCl. In contrast to the IDF standard procedure [13], samples were not directly used for HPLC analyses, but were dried as reported by some authors [8, 16, 26]. Purified eluates (200 μL) were gently vacuum-dried using a Waters Pico·Tag™ workstation, and dried samples were dissolved in 200 μL of a freshly prepared mixture of water, acetonitrile, and formic acid (94.8:5:0.2) before HPLC analysis. Duplicate sample hydrolysates were used for drying.

The same HPLC equipment and in particular the same HPLC column was used as described for the analysis of β-Lg to avoid the need for recurrent replacement of column and to improve the flexibility of HPLC equipment. Samples were filtered through 0.20 μm Minisart RC 4 filters (Sartorius). Column eluates were monitored at 280 nm using a Waters 2489 UV/VIS Detector. In contrast to other authors [7, 8, 16, 26, 27], two separated mobile phases were used, which were continuously mixed by the used HPLC multisolvent delivery system during isocratic separation of furosine. Solvent A consisted of 0.2% formic acid in 5 mmol·L^{-1} sodium heptane sulphonate (Sigma) solution (prepared from a 50 mmol·L^{-1} stock solution), and solvent B was 100% acetonitrile. After flushing the column for 20 min with solvent B, the initial conditions were set until a stable baseline was observed (~ 30 min): 89% solvent A and 11% solvent B. Samples (20 μL) were applied onto the column and eluted at 35 °C at a flow rate of 0.35 mL·min^{-1} using isocratic conditions. Intervals for sample injections were 20 min, each sample was injected in duplicate. Furosine (NeoMPS PolyPeptide Laboratories Group, Strasbourg, France) was used as standard. Calibration (5, 10, 20, 40, 80, and 160 pmol·20 μL^{-1} injection volume) was performed by plotting peak area versus picomole of furosine. Concentrations of furosine were displayed as picomole, which were subsequently converted to the results given in mg·100 g^{-1} protein (Tab. I). As protein results were needed for the calculation, total

nitrogen content of milk samples was determined using the Kjeldahl method after mineralization [12].

2.4. SDS-PAGE of milk proteins

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyse total milk proteins as well as acid-soluble whey proteins. Milk samples (100 μL) and acid whey after isoelectric precipitation of casein and denatured whey proteins (100 μL) were directly diluted (1:5) with sample buffer (2% SDS in 50 mmol·L^{-1} Tris-HCl buffer, pH 7.2), mixed with 5 μL 2.6 mol·L^{-1} dithiothreitol (DTT) solution, and heated in a thermoblock for 10 min at 100 °C. After cooling to room temperature, 5 μL DTT solution was added as a reducing agent again. Samples were mixed with 10% (v/v) bromophenol blue solution (0.1% bromophenol blue and 8.7% glycerol), and proteins were separated by SDS-PAGE (15% T) according to a method described recently [19].

2.5. Alkaline PAGE of whey proteins

Alkaline PAGE was used to separate acid-soluble whey proteins. Acid whey (see Sect. 2.2) (100 μL) was directly diluted (1:5) with sample buffer (10 mmol·L^{-1} Tris and 77 mmol·L^{-1} glycine buffer, pH 8.3) and mixed with 10% (v/v) bromophenol blue solution. Native PAGE (12.5% T) was performed using a dual cooled vertical slab gel electrophoresis unit SE 600 (Hoefer Scientific Instruments, San Francisco, CA, USA) as reported recently [19].

3. RESULTS AND DISCUSSION

3.1. Electrophoresis of milk proteins

In this study, electrophoresis of milk proteins was performed as a rapid and reliable screening technique to assess the heat load of milk and to distinguish different categories of heat-treated milk samples. The electrophoretic separation of the total milk proteins obtained by SDS-PAGE analysis of ESL milk samples was just to confirm that all milk samples analysed had almost the same electrophoretic pattern. After cooking milk samples (for 10 min at 100 °C) in the presence of SDS and DTT, intense casein bands, strong β-Lg, and weak α-La bands were detected in all samples, irrespective of the heating processes during manufacturing (results not shown). However, SDS-PAGE patterns of the acid whey of ESL milk samples showed distinct differences between individual samples. Since precipitated casein (including also the heat-denatured whey proteins) had been removed by centrifugation, acid whey showed just the acid-soluble whey proteins soluble at pH 4.6 (results not shown). In accordance with the results obtained by SDS-PAGE, the separation of non-denatured whey proteins by alkaline PAGE demonstrated even more clearly the differences between samples (Fig. 1). Figure 1 shows the electrophoretic patterns of selected milk samples from different categories of heat treatment (raw, pasteurized, ESL, and UHT milk). It is obvious that band intensity of acid-soluble whey proteins decreased corresponding to an increased heat load of milk samples analysed (from raw milk to UHT milk). The main whey proteins involved are in order of decreasing heat stability: α-La > β-Lg > BSA > immunoglobulins [1, 4, 17]. Thus, depending on heat stability of whey proteins, individual whey protein fractions decrease as a consequence of heating processes and can be used as a reliable tool to study the heat load of commercial milk samples. All the more, considering the fact that costs for acetonitrile needed for HPLC analyses of acid-soluble β-Lg and furosine have tremendously increased during last months, electrophoresis proved to be a high-throughput, cost-effective, and reliable screening method

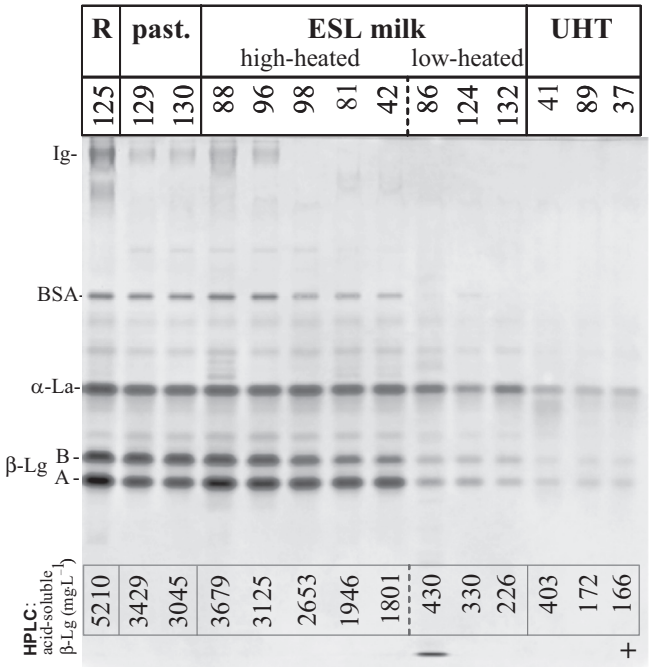

Figure 1. Alkaline polyacrylamide gel electrophoresis of whey protein fractions soluble at pH 4.6 from different categories of heat-treated milk: raw milk (125), pasteurized milk (129 and 130), low-heated (88, 96, 98, 81, and 42) and high-heated (86, 124, and 132) ESL milk, and UHT milk (41, 89, and 37) samples. Ig (immunoglobulins), BSA (bovine serum albumin), α-lactalbumin (α-La), and β-lactoglobulin (β-Lg) were separated depending on their negative charge.

to differentiate between low-heated and high-heated ESL milk.

3.2. RP-HPLC analysis of acid-soluble β-lactoglobulin in milk samples

As electrophoretic patterns of acid-soluble whey proteins were used just for screening purposes, an HPLC method for the quantitative determination of acid-soluble β-Lg was established. Figure 2 shows the RP-HPLC chromatogram of a standard mixture of α-La and β-Lg using a Symmetry 300™ C_{18} column (Waters), which enabled an acceptable resolution of whey proteins within 22 min superior to that reported in the IDF standard procedure [14]. Linearity of calibration was appropriate (R^2 = 0.999) in the range of 0.2–3.2 μg, corresponding directly to the range of most analysed sample contents (200–3200 mg·L^{-1} milk). The precision of the entire procedure including sample preparation and RP-HPLC analysis (same day) was evaluated on milk samples having low and high β-Lg contents. The relative standard deviation (RSD) was 1.91% obtained on a UHT milk sample with an average β-Lg content of 339.9 ± 6.5 mg·L^{-1} milk, and 0.26% on an ESL milk sample (2397.8 ± 6.2 mg·L^{-1}) (n = 8). Results of all milk samples analysed are given in Table I. The obvious difference between ESL milk samples of good and poor

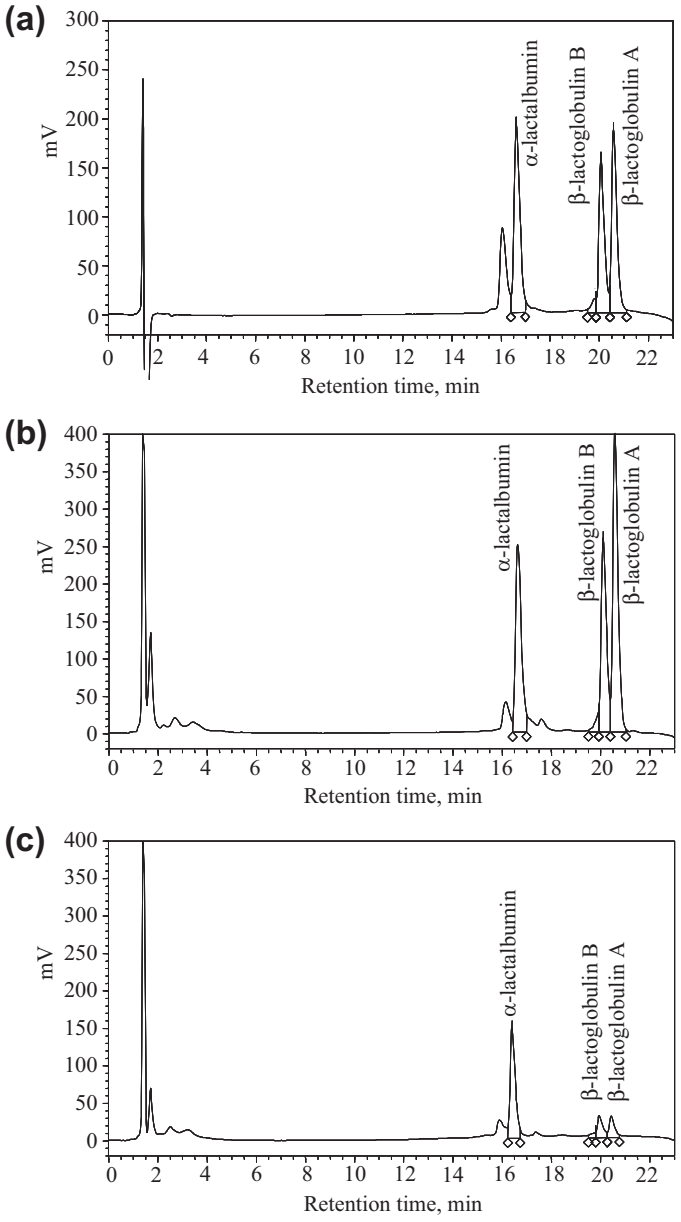

Figure 2. RP-HPLC chromatograms of a standard mixture of α-lactalbumin (0.8 μg) and β-lactoglobulin (1.6 μg) per 10 μL injection volume each (a), and of acid-soluble whey proteins in low-heated (b) and high-heated (c) ESL milk samples, having native β-Lg contents of 2792 and 260 mg·L^{-1} milk, respectively (Nos. 127 and 31).

quality is demonstrated in Figure 2. Compared to the results obtained by other authors [14, 21], a faster separation and a much more stable baseline were the most striking advantages of the HPLC column used in the present study.

3.3. RP-HPLC analysis of furosine in milk samples

A specific "Furosine dedicated" RP-HPLC C_8 column had been proposed by the FIL/IDF standard [13], whereas different types of C_{18} columns were reported for RP-HPLC by other authors [7, 8, 16, 23, 26, 27]. However, as furosine and β-Lg contents of many milk samples were to be analysed in alternating series, an effort was made to develop an RP-HPLC method for the analysis of furosine using the same column as for β-Lg, to avoid recurrent replacement of column during this study. Figure 3 shows the chromatogram of a furosine standard using a Symmetry 300™ C_{18} column (Waters), which enabled an excellent separation of furosine within 8 min superior to that reported in the IDF standard procedure within 22 min [13], and comparable to that of other authors [8, 16, 26]. However, in contrast to all available reports [7, 8, 16, 26, 27], two separated mobile phases were used, which were continuously mixed by the used HPLC multisolvent delivery system during isocratic separation of furosine. Continuous mixing of two separated solvents (solvent A was 0.2% formic acid in 5 mmol·L^{-1} sodium heptane sulphonate and solvent B was 100% acetonitrile) was of utmost importance to get a stable baseline, a proper resolution, and constant retention time of furosine peaks in different samples.

Linearity of regression line was appropriate (R^2 = 0.999) in the range of 5–160 pmol. Precision of the entire procedure (n = 32) including acid hydrolysis (n = 4), Sep-Pak® purification (n = 2), vacuum-drying in Pico·Tag™ workstation (n = 2), and RP-HPLC analysis (two injections each) was evaluated on an ESL milk sample with an average furosine content of 49.87 ± 0.55 mg·100 g^{-1} protein (RSD was 1.10%). Results of all milk samples analysed are given in Table I. The substantial difference between ESL milk samples of good and poor quality is shown in Figure 3.

3.4. Comparison of acid-soluble β-Lg and furosine contents in ESL milk samples

Acid-soluble β-Lg and furosine contents of all milk samples from different categories of heat treatment (raw, pasteurized, ESL, and UHT milk) analysed in this study are listed in Table I. Results were given in order of decreasing β-Lg contents of milk samples within each category, mean values for each category are compiled in Figure 4. As expected, pasteurized milk samples had high β-Lg (mean value = 3177 ± 288 mg·L^{-1}) and low furosine (9.9 ± 1.3 mg·100 g^{-1} protein) contents, whereas UHT milk conversely showed very low β-Lg (226 ± 67 mg·L^{-1}) and high furosine (204 ± 124 mg·100 g^{-1} protein) contents. Surprisingly, ESL milk samples had to be divided into two separate groups: ESL milk of good quality (45% of analysed samples) showed acid-soluble β-Lg contents > 1800 mg·L^{-1} and furosine contents < 40 mg·100 g^{-1} protein, whereas ESL milk of poor quality had low acid-soluble β-Lg (< 500 mg·L^{-1}) and high furosine contents (> 40 mg·100 g^{-1} protein), which was comparable to the excessive heat load of UHT milk (Fig. 4). This remarkable bisection of ESL milk samples can be seen even more clearly in Figure 5, where β-Lg contents of all analysed milk samples were plotted against furosine contents (R^2 = 0.856). Raw milk, pasteurized, and ESL milk samples of good quality were clustered on top and on the left side (having low furosine and high β-Lg contents), whereas ESL milk samples of poor quality and UHT milk samples were grouped on the bottom

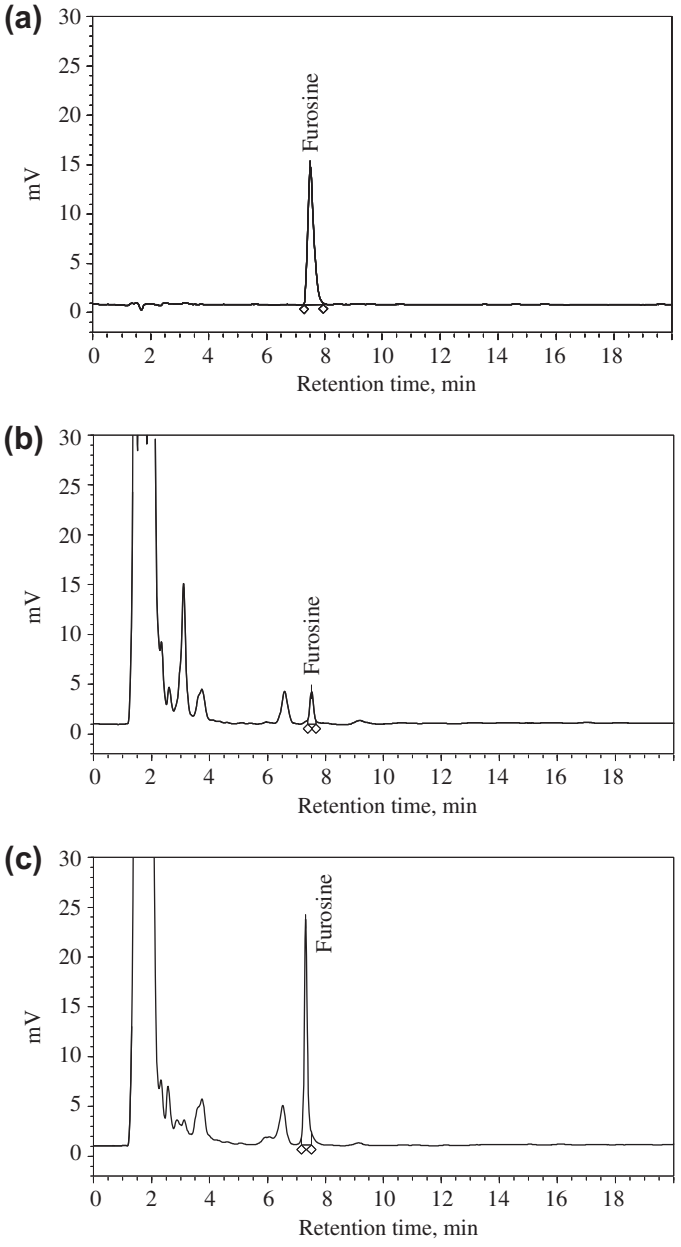

Figure 3. RP-HPLC chromatograms of a furosine standard with 80 pmol per 20 μL injection volume (a), and of furosine in low-heated (b) and high-heated (c) ESL milk samples, having furosine contents of 11.6 and 74.7 mg·100 g^{-1} protein, respectively (Nos. 127 and 31).

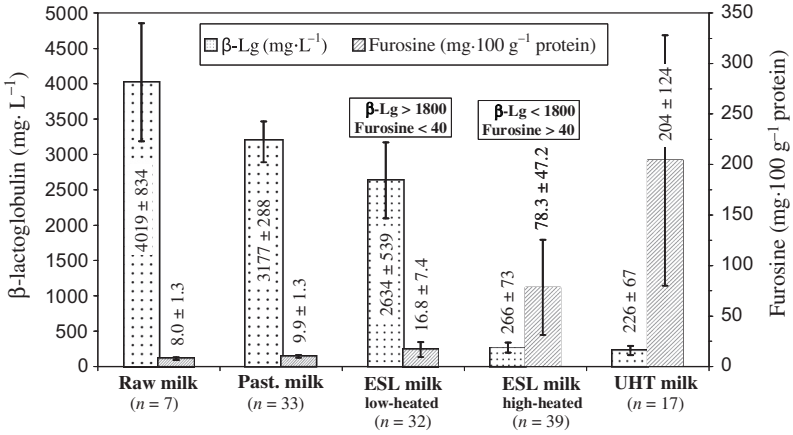

Figure 4. Mean values (± SD) for acid-soluble β-Lg (mg·L^{-1}) and furosine (mg·100 g^{-1} protein) contents in different categories of heat-treated milk samples taken from retail outlets in Austria (n = 128).

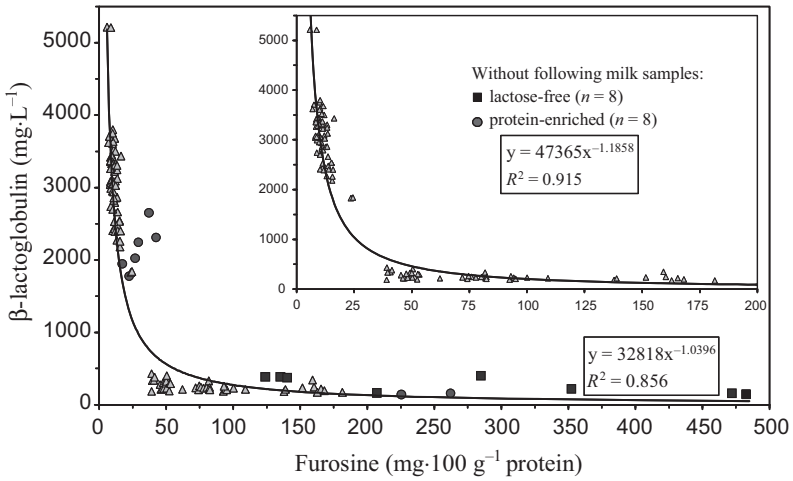

Figure 5. Relationship between acid-soluble β-Lg (mg·L^{-1}) and furosine (mg·100 g^{-1} protein) contents of milk samples taken from retail outlets in Austria (n = 128).

and on the right side of the graph (showing high furosine and low β-Lg contents). As protein-enriched and lactose-free milk samples partly did not fit the curve as perfect as the other samples, they were omitted to get a logarithmic relationship with an improved regression coefficient (R^2 = 0.915).

Figure 6 shows the relative distribution of acid-soluble β-Lg and furosine contents of all ESL milk samples analysed in this study (n = 71). ESL milk samples of good quality (β-Lg > 1800 mg·L^{-1}; 45%) were subdivided into four groups, taking into account some additional limits of 2000, 2500, and 3000 mg·L^{-1}. A minimum content of 2000 mg·L^{-1} had been proposed for high-pasteurized milk [3, 4], whereas 1800 mg·L^{-1} was discussed as threshold

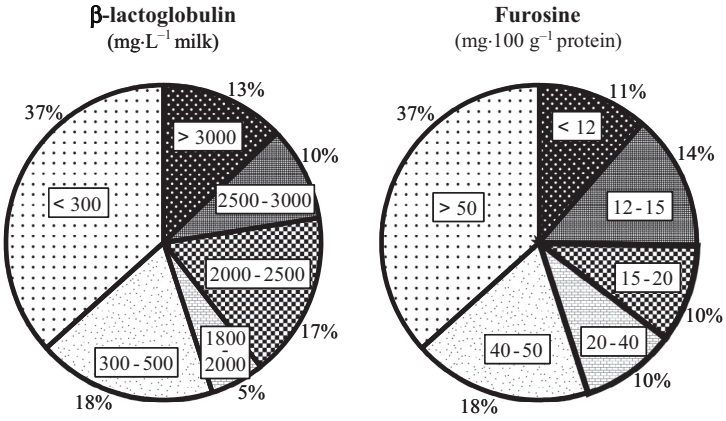

Figure 6. Relative distribution of acid-soluble β-Lg (mg·L^{-1}) and furosine (mg·100 g^{-1} protein) contents in ESL milk samples taken from retail outlets in Austria (n = 71).

level in Austria and in some other European countries due to the lack of obligatory limits regarding the heat load of ESL milk [9, 15]. As there were only four samples (5%) between these two suggested limits (Tab. I and Fig. 6), both would be appropriate as obligatory limits to control the heat load of ESL milk samples by regulatory authorities.

The relative distribution of furosine contents of all ESL milk samples reflected the above-mentioned remarkable bisection of ESL milk samples of different quality in a very similar manner (Fig. 6). ESL milk samples of good quality (furosine < 40 mg·100 g^{-1} protein; 45%) were also subdivided into four groups, taking into account the additional limits of 20, 15, and 12 mg·100 g^{-1} protein. Although a furosine content of 20 mg·100 g^{-1} protein had been suggested as upper limit for high pasteurization [3, 4], a limit of 40 mg·100 g^{-1} was defined in this study, because of the acid-soluble β-Lg contents of the respective ESL milk samples of good quality. Nevertheless, the reported upper furosine limit of 20 mg·100 g^{-1} protein for high pasteurization would very nicely fit to the results obtained in the present study, because there were only seven ESL milk samples in between 20 and 40 mg·100 g^{-1} protein. Moreover, five samples thereof were protein-enriched ESL milk, and only two samples (Nos. 49 and 18) had furosine contents of 24.5 and 23.6 mg·100 g^{-1} protein, respectively (Tab. I). Correspondingly, acid-soluble β-Lg contents were also very low in these two samples (1827 and 1801 mg·L^{-1}).

Obviously, ESL milk samples of poor quality had been manufactured using an indirect UHT process that had been reported to be cheaper (regarding investment and production costs) and more energy-efficient compared to the direct UHT heating techniques using injection or infusion systems. On the other hand, directly UHT-treated ESL milk suffers less heat damage than indirectly heated milk and can therefore be used in the production of ESL milk as an alternative to modern microfiltration techniques [10, 15, 18, 24, 25].

4. CONCLUSIONS

As ESL milk has shown a dramatic increase in Austria recently, and has been

widely accepted in many other European countries (e.g. Germany) in the meantime, the nutritional and organoleptic quality of this new category of liquid milk has to be controlled in the future. Since dairy companies are obviously not aware of the negative effects caused by an overheating of liquid milk, there is an urgent need for establishing obligatory threshold levels (limits) for ESL milk regarding TTIs (e.g. acid-soluble β-Lg, furosine, and lactulose). In any case, ESL milk should represent a milk product with taste and nutritional quality similar to pasteurized milk, but show the obvious benefits of longer shelf life. However, it must not be as highly heat-treated as UHT milk to fulfil the consumer's expectations regarding nutritional quality of this upcoming product. In striking contrast to these requirements, the present study showed that 55% of the analysed ESL milk samples had low acid-soluble β-Lg (< 500 mg·L^{-1}) and high furosine contents (> 40 mg·100 g^{-1} protein), which was comparable to the excessive heat load of UHT milk. Thus, there is an urgent need for an EU regulation to define obligatory limits for the tolerable heat load of ESL milk, which should be checked by regulatory authorities as soon as possible. In conclusion, results of the present study strongly support the proposed furosine content of 20 mg·100 g^{-1} protein as an upper heating limit for high-pasteurized ESL milk in general [3, 4, 6], whereas a higher limit of 40 mg·100 g^{-1} protein must be accepted for protein-enriched ESL milks. Nevertheless, acid-soluble β-Lg proved to be the more robust heat indicator for the assessment of heat load of unknown ESL milk samples, and the suggested β-Lg content of 1800 mg·L^{-1} (or maybe 2000 mg·L^{-1}) milk is therefore highly recommended as an upper heating limit for high-pasteurized ESL milk. Hereby, electrophoresis of whey proteins and HPLC of acid-soluble β-Lg and furosine offer high-throughput, cost-effective, and reliable tools to evaluate and control the heat load of ESL milk to minimize the loss of nutritional quality of milk with ESL in future.

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