Sensitive Determination of Lysinoalanine for Distinguishing Natural from Imitation Mozzarella Cheese[1]

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ABSTRACT

A new method of reverse-phase HPLC was used to determine the crosslinked amino acid lysinoalanine in natural Mozzarella cheese, dairy-based substitutes, and related ingredients. Commercial samples manufactured under known conditions or collected at the market were analyzed. The acid-hydrolyzed sample derivatized by 9-fluorenyl-methylchloro-formate was submitted to solid-phase extraction on an amino cartridge to extract selectively the lysinoalanine derivatives that were chromatographed under fluorescence detection.

Lysinoalanine was not found (<0.4 ppm in protein) in pasteurized milk, but 0.4 to 4 ppm ($\overline{X}$ = 1.7; n = 30) were present in natural Mozzarella cheese. Because of the ingredient characteristics and the more severe thermal processing conditions, the different types of processed cheese and imitation Mozzarella cheese exhibited much higher lysinoalanine contents, ranging from 15 to 421 ppm ($\overline{X}$ = 54; n = 29). Hence, a highly significant distinction between natural Mozzarella cheese and imitations, even those that did not contain added milk protein, could be achieved by the lysinoalanine index. Conversely, the furosine index distinguished the imitation products only when the quantity of reducing sugars allowed the early Maillard reaction to be extensive.

(**Key words**: lysinoalanine, high performance liquid chromatography, Mozzarella cheese, imitation Mozzarella cheese)

Abbreviation key: FMOC = 9-fluorenylmethyl-chloroformate, **FUR** = furosine, **LAL** = lysinoalanine, **SPE** = solid-phase extraction.

INTRODUCTION

The increased demand for different types of pasta filata cheese, such as traditional or low moisture Mozzarella (18, 21), makes the development of imitation products attractive because of their lower cost and longer storage stability (17, 29). Mozzarella is a fresh cheese of high quality produced from natural milk only, and, in several countries, this cheese is legally protected against the imitation products. Mozzarella cheese substitutes, used mainly in pizza and related foods, are of lower quality and are manufactured from mixtures of different ingredients by conventional in-vat cheese making or by heat blending in the presence of emulsifying salts, followed by melting or stretching (29, 31). An objective analytical method for detection is required because of the possibility of replacing natural Mozzarella cheese with its substitutes during food preparation or as a final product (32, 33).

Because types and proportions of the ingredients can vary greatly according to the type and the desired functional properties of the imitation cheese, Mozzarella imitations cannot be identified on the basis of the gross chemical composition when only dairy ingredients are used. Furosine (**FUR**), arising from acid hydrolysis of the Amadori compound (10), is a suitable marker of ingredients that contain lactose and are prepared under thermal conditions that initiate early stages of the Maillard reaction (28). In Italy, 12 mg/100 g of protein is the upper concentration of FUR allowed by law in fresh pasta filata cheese to avoid illegal cheese made from reconstituted NDM (22). Nevertheless, imitation Mozzarella cheese can be manufactured from ingredients that do not contain significant amounts of lactose or are not submitted to severe thermal processes (17, 20). Under such conditions, the Maillard reaction is restricted, and FUR no longer represents a distinctive parameter.

Rennet casein and caseinate are ingredients that are typical of imitation Mozzarella cheese manufactured with different procedures (17, 33). Tunick et al. (32) applied differential scanning calorimetry to detect caseinate in Mozzarella cheese stored at temperatures <18°C. Process-induced molecules, arising during industrial preparation of casein or thermal treatments of imitation cheese, might be assumed to be

specific markers. Crosslinked AA form during heating of protein, mainly at neutral or subalkaline pH, even under mild thermal treatments and without reducing sugars (4, 8, 11). These unnatural AA occur in commercial casein and may form during the processing of imitation cheese as well, but few, if any, are expected in natural cheese. Because of its nutritional and toxicological significance (4, 11), lysinoalanine (**LAL**) represents the most extensively studied crosslinked AA. Lysinoalanine arises from the nucleophilic addition of the ε-amino group of Lys to the dehydroalanine residues, which are produced via the β-elimination reaction from Cys or P-Ser residues (15). The highest concentrations of LAL occurred in commercial caseinate, and lower concentrations of LAL occurred in milk treated under high heating conditions and in protein-rich beverages (4). The literature (9, 14, 25) reports very small or unquantifiable amounts for natural cheese, processed cheese, and rennet casein. The values of >50 ppm in protein of some cheese products were related to presence of caseinate (14). Methods based on TLC (30), ion-exchange chromatography (13), GLC (2), and HPLC (34) have been proposed for LAL determination. Although 1 to 5 ppm of LAL in protein were reported to have been detected (13), only data of >10 to 15 ppm were quantified (4) and not free of interference (25).

The present investigation was conducted to ascertain whether the LAL index is a useful parameter for distinguishing between natural Mozzarella cheese and the imitation products, even those without added caseinate. To accomplish this objective, a very sensitive and selective method capable of quantifying LAL concentrations <15 ppm in complex foods was studied first. The LAL contents of ingredients and end products were determined for natural Mozzarella cheese and its substitutes, both of which were manufactured under known conditions. The possible ranges of the LAL values for these two classes of products, including several commercial samples, were further defined. Moreover, FUR was always determined in order to compare the extent of the Lys crosslinking with that of the initial stages of the Maillard reaction.

MATERIALS AND METHODS

Dairy Samples

All of the cheese products were from the major European brands, which were manufactured with dairy ingredients only. Products were collected at cheese factories, in which formulations and processing conditions were controlled especially for heat treatment (samples of known origin). Other samples were purchased (market samples).

Natural Mozzarella cheese. Samples of known origin of full fat Mozzarella cheese of traditional (52 to 60% moisture; n = 5) or low moisture (45 to 52% moisture; n = 2) types were produced by three manufacturers under conventional industrial conditions (20) using milk pasteurized at 75 to 85°C for 10 to 20 s. Rennet curds cultured (pH 5.1 to 5.2) or directly acidified with citric acid (pH 5.8 to 5.9) were submitted to continuous stretching and molding at temperatures ranging from 56 to 61°C for 10 to 20 min, depending on the type of Mozzarella, followed by cooling at 4 to 10°C. The market samples of full fat Mozzarella cheese of traditional (n = 15) or low moisture (n = 8) types included 20 brands.

Casein. Commercial samples of rennet casein (n = 9) and calcium or sodium caseinate (n = 6) with protein content ranging from 75 to 88% (wt/wt) were of different origins (France, Denmark, Russia, and New Zealand) and were supplied by Italian importers.

Processed cheese and imitation Mozzarella cheese. Samples of processed cheese of known origin were produced by two manufacturers. Formulations included 5 to 10% of milk powder or whey powder for the spreadable (n = 3) types but not for the sliced (n = 2) types. Emulsifying salts (1.5 to 3.0%, wt/wt), represented by polyphosphate and citrate for the spreadable type and by citrate only for the sliced type, were always added to the usual dairy ingredients, which in most cases included 3 to 5% (wt/wt) of rennet casein. Melting was performed at 80 to 98°C for 15 to 20 min in a batch cooker and, for the spreadable samples, was followed by sterilization in a heat exchanger at 110 to 130°C for 3 to 5 min and by cooling at 15°C. Samples of low moisture imitation Mozzarella cheese (n = 3) of known origin were produced by two manufacturers; 4 to 5% of rennet casein and 2 to 3% of citrate were included as ingredients. The mix at pH 5.7 to 5.9 was submitted to batch heat processing at 65 to 70°C for 10 to 20 min, followed by continuous stretching at 57 to 62°C for 10 to 20 min, and by cooling at 4 to 8°C. A sample of imitation Mozzarella cheese of known origin that was of traditional type (54% moisture) was manufactured from milk with 0.5% (wt/vol) of calcium caseinate added, and the sample was processed conventionally. The market samples were processed cheese of spreadable (n = 5) or sliced (n = 7) types and imitation Mozzarella cheese of traditional (n = 2) and low moisture (n = 6) types comprising 15 brands.

Blended cheese products. A cultured rennet curd for Mozzarella manufacture was mixed in different proportions with imitation Mozzarella cheese of low moisture type. The usual stretching and molding at 60°C in mechanical batch equipment were performed at an industrial manufacturer.

Cheddar cheese products. Market samples of natural Cheddar cheese (n = 2) and processed Cheddar cheese (n = 3) were from 5 brands.

Other dairy products. Pasteurized bulk cheese milk (n = 3) was sampled at the factories. Samples of packaged pasteurized milk (n = 2), NDM of low heat (n = 1) and medium heat (n = 2) classes, and rennet whey powder (n = 1) were of market origin.

Chemicals, Reagents, and Equipment

All chemicals for 9-fluorenylmethylchloroformate (**FMOC**) derivatization, solid-phase extraction (**SPE**), and HPLC were of high purity or HPLC grade. Synthesized LL, DL-LAL was purchased from Bachem (Bubendorf, Switzerland). The FMOC reagent, prepared daily by dissolving 5 mg of FMOC (Pierce, Rockford, IL)/ml of acetonitrile, and the derivatization buffer, consisting of 0.4 M borate buffer at pH 12, were used for the sample derivatization. A conditioning solvent (acetonitrile:0.1 M borate buffer at pH 7.2 = 1:4, vol/vol) and an eluting solvent (acetonitrile:0.2 M borate buffer at pH 9.0 = 1:1, vol/vol) were used for SPE on an amino cartridge (500 mg/3 ml; Baker, Phillipsburg, NJ) using either a manual or automated procedure with an Aspec XL (Gilson, Villiers Le Bel, France). The HPLC elution solvents were prepared from stock solution 1 (0.5% tetrahydrofuran and 0.1% ethyl acetate in 30 mM potassium acetate (vol/vol/vol) and stock solution 2 (80% acetonitrile in 100 mM sodium acetate; vol/vol). The stock solutions were mixed (vol/vol) for preparing the elution solvent A (solution 1:solution 2 = 70:30) and the elution solvent B (solution 1:solution 2 = 25:75). The elution solvent C was 100% of stock solution 2. The HPLC was carried out with a Waters 625-LC System (Waters, Milford, MA) provided with an automatic injector (model 234; Gilson) and a fluorescence detector (model F1080; Hitachi, Tokyo, Japan). The chromatographic column (Amino-Quant, 200 × 2.1 mm i.d.; Hewlett-Packard, Waldbronn, Germany) was attached to an ODS-Hypersil C18 guard-column cartridge (5 μm, 20 × 2.1 mm i.d.; Hewlett-Packard). Chromatographic data were processed by 860™ software from Waters operating on a MicroVAX II (Digital, Maynard, MA) computer.

LAL Determination

The procedure for LAL determination in dairy products included sample acid hydrolysis, FMOC derivatization of the amino compounds, selective SPE of the FMOC and LAL derivatives, and subsequent reverse-phase HPLC with fluorescence detection.

Sample hydrolysis and FMOC derivatization. An amount of finely chopped or powdered sample,

corresponding to approximately 10 mg of protein, was weighed in a 35-ml glass vial provided with mininert valve cap (Alltech, Deerfield, IL) to which 30 ml of 6 M HCl were added, sealed under vacuum, and kept at 110°C for 23 h. The hydrolysate was submitted to FMOC derivatization according to the method of Einarsson et al. (7) modified to achieve the highest fluorescence response of LAL molecule. Five milliliters of the filtered acid hydrolysate were dried under vacuum at 45°C in a 15-ml conical flask through a Rotavapor (Büchi, Flawil, Switzerland), and the residual material was dissolved with 200 μl of derivatization buffer. Fifty microliters of this solution were transferred to a 2-ml glass vial to which 100 μl of water, 50 μl of derivatization buffer, and 200 μl of FMOC reagent were added; the closed vial was kept at room temperature (20 to 25°C) for 30 min. The derivatized sample solution was stable for 24 h at room temperature or for several days at –20°C.

SPE. An aliquot of 100 μl of derivatized sample solution was subjected to SPE on an amino cartridge by the procedure reported in Table 1. The eluted sample solution proved to be stable for some days at room temperature.

Separation by reverse-phase HPLC. An aliquot of 20 μl of eluted sample solution was injected onto the HPLC column, at 27 ± 0.5°C, and the separation was carried out by the linear elution gradient of Table 2 at a flow rate of 0.5 ml/min. When no SPE was utilized, the derivatized sample solution was diluted 15-fold with acetonitrile and water (1:1, vol/vol) prior to injection. Fluorescence detection was performed with excitation at 266 nm and emission at 310 nm.

LAL quantification. The two peaks of the LAL-FMOC derivatives were integrated on the baseline, and the sum of the area counts was computed. A calibration curve was obtained for an acid-hydrolyzed cheese that was free of LAL and supplemented, before drying, with different amounts of synthesized LAL standard solution (50 nmol/ml of water) in order to inject 5, 10, 50, 100, 500, 1000, and 10,000 pmol of LAL. These values corresponded to LAL concentrations in protein ranging from 1 to 2000 ppm. Because of the linearity of the response in this wide range of values, a single-point external standard quantification with one standard sample (0.1 or 1 pmol injected) was usually adopted. To account for differences in gross chemical composition of the samples, LAL was based on protein (parts per million).

Other Chemical Analyses

Furosine was determined on acid-hydrolyzed samples by direct ion-pair reverse-phase HPLC and UV detection according to method of Resmini et al. (27) and was expressed as milligrams/100 g of protein.

TABLE 1. Procedure for solid-phase extraction of lysinoalanine derivatives on an amino cartridge.

Extraction step	Type of solvent	Volume of solvent	Proximate flow rate[1]	Cartridge drying[2]
		(ml)	(ml/min)	
Prewetting	ACN[3]	10	10	–
Conditioning	ACN:0.1 M borate buffer (pH 7.2), 1:4 (vol/vol)	10	10	–
Sample loading	Derivatized sample solution	0.1	0.5	+
Washing	ACN:Water, 1:4 (vol/vol)	6	3	+
Washing	ACN:Water 1:1 (vol/vol)	5	3	+
Eluting	ACN:0.2 M borate buffer (pH 9.0), 1:1 (vol/vol)	1.5	1	+

[1]Flow rate ±20%.

[2]Drying performed in few seconds with 20 ml of air after the extraction step was applied (+) or not applied (–).

[3]Acetonitrile.

The protein content of the dairy samples was determined by Kjeldahl (N × 6.38) according to the method of the International Dairy Federation (16).

RESULTS AND DISCUSSION

Determination of LAL by Reverse-Phase HPLC

Under the conditions described for FMOC derivatization, the synthesized LAL molecule formed two derivatives that were separated in two different chromatographic peaks with retention times of 25 and 26 min, respectively, without (Figure 1A) or with (Figure 1B) adoption of sample SPE before HPLC. The presence of two primary amino groups and one secondary amino group in the LAL molecule made possible the formation of different FMOC derivatives, but the isomers (15) were unlikely to be separated using the conditions described. The precolumn reaction of LAL with dansyl-chloride led to three derivatives (34). Because of the strong hydrophobicity of FMOC molecule, if two amino groups were derivatized in the same LAL molecule, the resulting double derivative could be hardly eluted from the stationary phase by the chosen solvents. In any case, the retention time of the double derivative would be much higher than that of the single derivative. If the secondary amino group were derivatized, the resulting single derivative would have a residual polarity rather different from that of the single derivative of the α-amino group, and an earlier retention time would be expected. Only the single derivatives of the two α-amino groups could explain two peaks closely eluted. In this case, a comparable fluorescence response could be assumed for the two peaks and the sum of the areas computed.

Although LAL was eluted in a relatively clean area of the chromatogram, interfering peaks with retention times slightly different from those of LAL were sometime observed in samples without LAL when no SPE was adopted (Figure 1A; pattern a). In a recent study by Pellegrino and Resmini (1995, unpublished), SPE was introduced on an amino cartridge to purify dairy samples that were derivatized with FMOC. The purification of the FMOC and LAL derivatives achieved by this technique is shown in Figure 1B. The interfering compounds were completely removed (pattern c), and the sample was enriched in LAL (pattern d); the closest significant peak (Lys with retention time of 21 min) was eluted 4 to 5 min before LAL peak.

This purification was accomplished by a selective extraction of the FMOC and LAL derivatives at pH 7.2. At this pH, the LAL molecule is tightly retained, mainly by the NH_2 groups of the cartridge, through polar interactions, allowing an exhaustive washing of the cartridge with mixtures of acetonitrile and water (Figure 2A). At pH >9.0 and salt concentration of

TABLE 2. Linear eluting gradient for reverse-phase HPLC of lysinoalanine derivatives.

Time	Eluting solvents		
	A	B	C
(min)		(%)	
0	100	0	0
1	70	30	0
15	62	38	0
30	62	38	0
31	0	100	0
32	0	0	100
36	0	0	100
37	100	0	0
45	100	0	0

0.2 *M*, the molecule was no longer ionized, and hydrophobic interactions between the FMOC moiety of the LAL derivatives and the propyl chains of the cartridge prevailed, allowing complete elution in presence of high amounts of acetonitrile (Figure 2B). More than 90% of the LAL was recovered in the third 0.5 ml of elution solvent (Figure 3) and approximately 95% was recovered in the first 1.5 ml, which represented the adopted elution volume.

Contrary to observations for the synthesized molecule, in dairy samples, the two LAL derivatives were always comparable for peak area, giving characteristic and well-recognizable twin peaks (Figure 4). In the synthesized molecule, FMOC derivatization might not be complete for one α-amino group, but further investigation is needed. Very low concentrations of LAL were determined without interference in every dairy product after SPE (Figure 5); the minimum detectable amount was <2 fmol injected corresponding to <0.4 ppm in protein. The linearity of
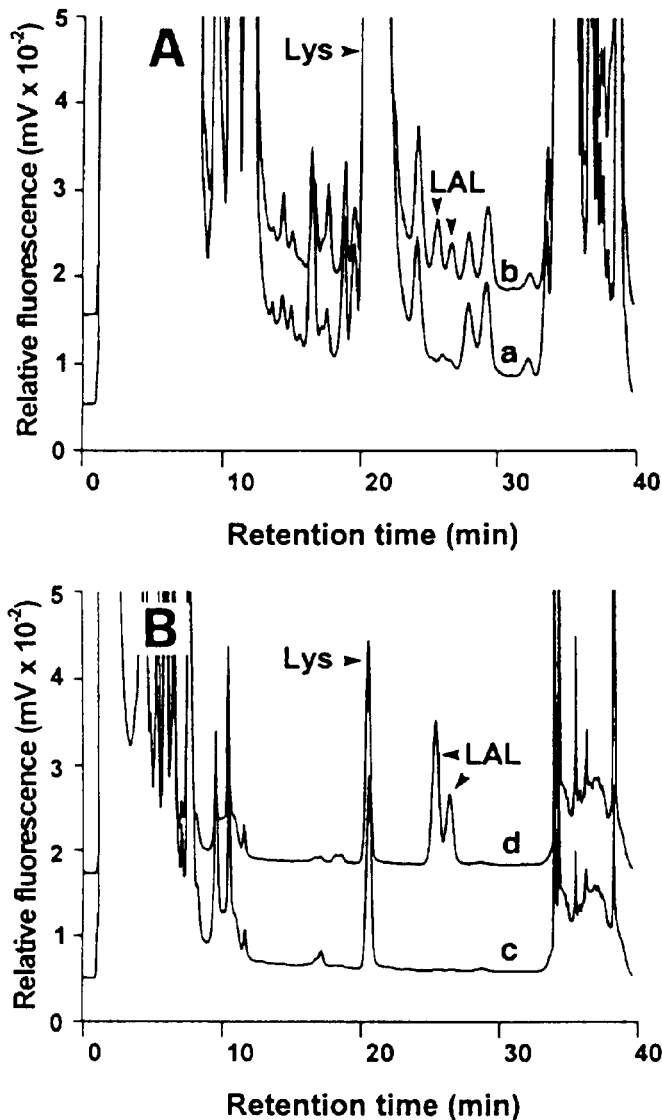

Figure 1. Reverse-phase HPLC patterns of natural Mozzarella cheese hydrolysate derivatized with 9-fluorenylmethylchloroformate not submitted (A) or submitted (B) to solid-phase extraction without (a, c) or with (b, d) addition of synthesized lysinoalanine (LAL) standard solution to hydrolysate (1 pmol injected).
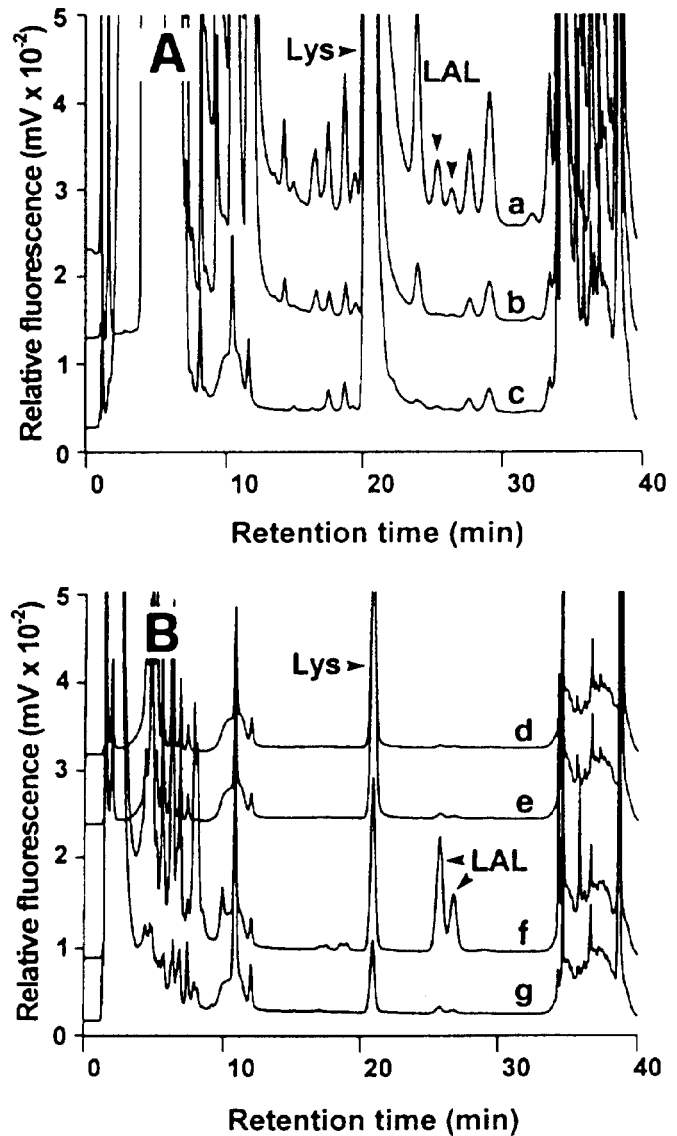
Figure 2. Reverse-phase HPLC of washed (A) and eluted (B) fractions obtained by solid-phase extraction of derivatized Mozzarella cheese hydrolysate with added synthesized lysinoalanine (LAL) standard solution (0.8 pmoles injected). The patterns refer to loaded sample (a), acetonitrile:water = 1:4 (vol/vol) (b), acetonitrile:water = 1:1 (vol/vol) (c), and subsequent 0.5-ml eluted fractions of acetonitrile:0.2 *M* borate buffer (pH 9.0) = 1:1 (vol/vol) (d, e, f, and g).

TABLE 3. Lysinoalanine (LAL) and furosine (FUR) in different types of heat-treated milk and natural Mozzarella cheese.[1]

Type of product	Origin[2]	n	LAL		FUR	
			(ppm in protein)		(mg/100 g of protein)	
			$\overline{X}$	Range	$\overline{X}$	Range
Heat-treated milk						
Pasteurized, bulk[3]	K	3	. . .	<0.4	6.1	6.0– 6.2
Pasteurized, packaged	M	2	. . .	<0.4	6.4	6.2– 6.5
Natural Mozzarella cheese						
Traditional	K	5	1.0	0.6–1.2	5.5	4.7–6.2
Traditional	M	15	2.1	0.4–4.0	7.3	5.3–11
Low moisture	K	2	1.0	0.9–1.1	6.8	6.2–7.4
Low moisture	M	8	1.8	0.7–3.8	12	5.8–23

[1]Means of duplicate analyses.

[2]K = Samples of known origin; M = samples collected at the market.

[3]Milk from the cheese vat.

the response was assessed in the range from 5 fmol to 10 pmol injected (r = 0.998). The analysis of three dairy samples containing 1.5, 44, and 102 ppm of LAL replicated on 3 different d gave a residual standard deviations of 4.5, 2.2, and 6.1%, respectively.

LAL in Dairy Products

The possible formation of LAL during heat treatments of milk and curd in Mozzarella cheese making was evaluated first. The LAL and FUR in heat-treated milk and in natural Mozzarella cheese are shown in Table 3. Less than 0.4 ppm of LAL was found in pasteurized bulk milk sampled from cheese vats or from retail packages. Others (5, 12) did not find LAL in pasteurized milk. The LAL content of natural Mozzarella cheese ranged from 0 to 4 ppm ($\overline{X}$ = 1.7; n = 30) and <1.2 ppm in the samples of known origin ($\overline{X}$ = 1.0; n = 7). Because no LAL formed during heat treatment of the cheese milk, the small amount of LAL in the samples of Mozzarella cheese of known origin could only arise from heating curd dur-
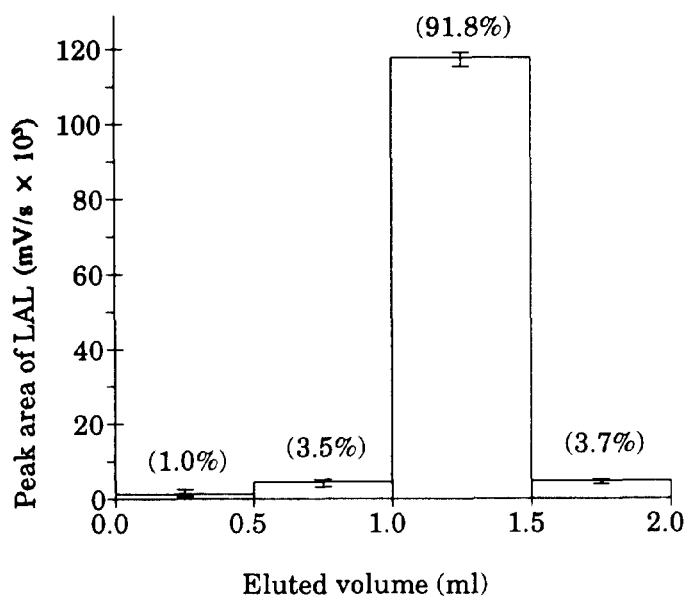

Figure 3. Elution of lysinoalanine (LAL) from the cartridge and mean percentage of recovery (in parentheses) in the subsequent 0.5-ml eluted fractions of acetonitrile:0.2 M borate buffer (pH 9.0) = 1:1 (vol/vol). Each bar represents the mean of three elutions.
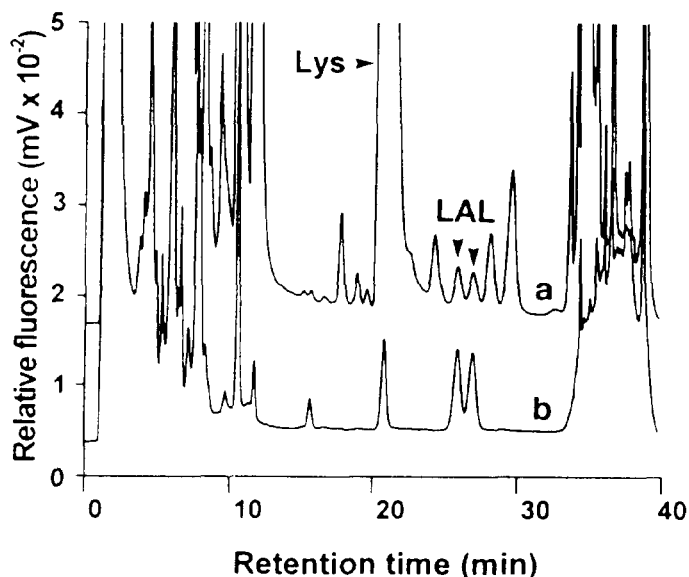

Figure 4. Reverse-phase HPLC patterns of a caseinate sample containing 164 ppm of lysinoalanine (LAL) in protein not submitted (a) or submitted (b) to solid-phase extraction.

TABLE 4. Lysinoalanine (LAL) and furosine (FUR) in commercial samples of casein and NDM.[1]

Type of product	n	LAL		FUR	
		(ppm in protein)		(mg/100 g of protein)	
		$\overline{X}$	Range	$\overline{X}$	Range
Casein					
Rennet casein	9	114	21–178	15	6.2–37
Calcium caseinate	5	977	128–2992	9.1	0–17
Sodium caseinate	1	...	5390[2]	...	7.6[2]
NDM					
Low heat	1	...	4.9	...	79
Medium heat	2	6.6	4.4[2]–8.9	768	136–1400[2]
Whey powder	1	...	2.9		1292

[1]Means of duplicate analyses.

[2]Values obtained in one sample of sodium caseinate and one sample of medium-heat NDM, both stored for long periods under warm conditions.

ing stretching and molding. The higher amounts of LAL in some commercial samples were probably related to usage of NDM as the starter medium in cheese making, as supported by the corresponding concentrations of FUR, which were higher (up to 23 mg in market samples) than those in the samples of known origin (4.7 to 7.4 mg).

As previously mentioned, casein is an important ingredient in formulation of Mozzarella substitutes. In rennet casein and caseinate samples, LAL contents
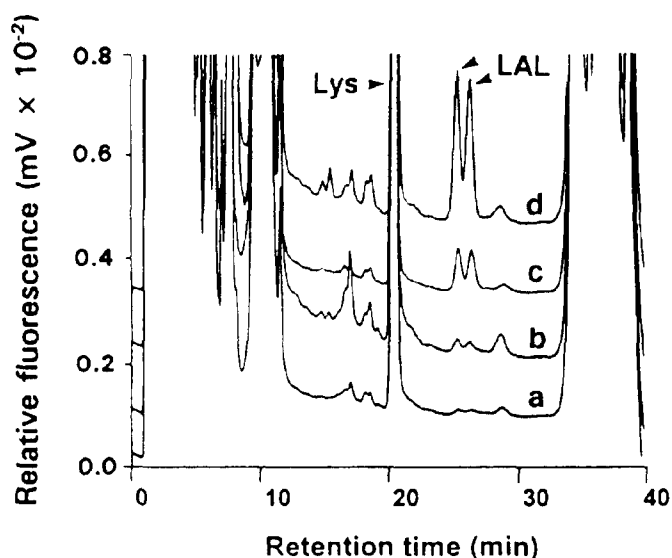

Figure 5. Reverse-phase HPLC of different dairy samples previously submitted to solid-phase extraction. The patterns refer to natural Mozzarella cheese containing <0.4 ppm (<1.6 fmol injected) (a) and 1.5 ppm of lysinoalanine (LAL) (b), low heat NDM containing 5.7 ppm of LAL (c), and processed cheese containing 22 ppm of LAL (d). All LAL values are expressed as concentrations in protein.

ranged from 21 to 178 ppm and from 128 to 5390 ppm, respectively (Table 4). High pH of caseinate accounted for these high contents of LAL (4, 9, 14). Because the LAL concentrations were from 20 to 5000 times higher than those occurring in Mozzarella cheese or in heat-treated milk, LAL represents a molecular marker for commercial casein, including rennet casein. Conversely, FUR, which is a well-established characterizing molecule of NDM (26, 28), was usually present in casein at very low concentrations. Concentrations of LAL were highest when the FUR content was low and vice versa, as clearly shown by samples of caseinate and NDM after long-term storage (Table 4). Competition between dehydroalanine residues and lactose toward the ε-amino group of Lys has been reported to occur in protein systems (4, 6), and the Maillard reaction prevails when a sufficient amount of reducing sugar is present. Because of the comparable activation energies of the two reactions (1, 4), prevalence of FUR formation over LAL formation might be related to higher chemical availability of lactose than of protein-bound dehydroalanine. However, coupling of the two indicators allowed the thermal history of a dairy product to be described in every case.

Because of the meltability and other functional properties, some types of processed cheese can substitute for natural Mozzarella in food preparations (33). Also, formulation and thermal treatments of these substitutes are sometimes close to those of imitation Mozzarella cheese. Therefore, processed cheeses were included in the present investigation. Processed cheese and imitation Mozzarella cheese exhibited LAL contents ranging from 15 to 421 ppm (Table 5); the mean value of 54 ppm (n = 29) was about 50-fold that of natural Mozzarella.

When no addition of casein or milk protein was made in the manufacture of Mozzarella substitutes,

the composition of the food system and the heating conditions were sufficient to produce a mean amount of LAL that was about 20-fold of that occurring during natural Mozzarella cheese making. The temperature reached by Mozzarella curd during stretching is about 57°C (29) and cannot exceed 62°C because of the risk of structure modification and collapse of protein fibers (21). In contrast, imitation Mozzarella cheese prior to stretching is submitted to heat blending at 65 to 70°C for 10 to 20 min or at higher temperatures for a melting process. Moreover, concentration of reducing sugars, which protect against Lys crosslinking (6), is kept as low as possible (18). Finally, micelle disaggregation promoted by the emulsifying salts (3) might increase the chemical availability of reactive groups of protein, thus enhancing the AA crosslinks and possibly explaining why the LAL index always represented a distinguishing parameter between natural and imitation Mozzarella cheeses, despite lack of added protein in formulation.

As expected, LAL increased in the end product when SNF were partly replaced with caseinate and much less with rennet casein, which is the most prevalent protein ingredient. The 90 and 360 ppm of LAL found in two samples of imitation Mozzarella cheese by Fritsch and Klostermeyer (14) were attributed to use of caseinate. Because of the low concentration (3 to 20 mg) of several casein-based imitation products, the FUR index represented a distinguishing parameter only when milk powder or whey powder was included in formulation, such as in spreadable processed cheese.

The reliability of the analytical characterization achieved by LAL determination is described in Figure 6. The highest LAL value predicted at the 3× standard deviation confidence level of natural Mozzarella cheese was 5.5 ppm; 15 ppm was the lowest concentration found for substitutes. This distinction was possible only when LAL was correctly quantified in at least the range from 1 to 20 ppm. Figure 6 also shows two cheese products obtained by blending during warm stretching of a rennet curd of natural Mozzarella (<0.4 ppm of LAL) with an imitation Mozzarella cheese (38 ppm of LAL) in the proportions 9:1 and 7.5:2.5 (wt/wt), respectively. The LAL concentrations were as expected and confirmed that only a very low amount of Lys crosslinking takes place during curd stretching. Hence, a rather small amount of imitation product blended together with natural Mozzarella cheese can be detected. This blending technique is being adopted by some manufacturers (31) to obtain a Mozzarella-like cheese product that can hardly be distinguished from the natural product.

TABLE 5. Lysinoalanine (LAL) and furosine (FUR) in different types of processed cheese and imitation Mozzarella cheese.[1]

Product type and origin[2]	Addition of protein[3]	n	LAL (ppm in protein)		FUR (mg/100 g of protein)	
			$\bar{X}$	Range	$\bar{X}$	Range
Processed cheese						
Spreadable						
K	NA	1	. . .	22	. . .	221
K	RC	2	63	60–66	230	180–281
M	NA	4	149	20–421[4]	228	3.0[4]–522
M	MP	1	. . .	50	. . .	446
Sliced						
K	RC	2	15	15–16	31	24–38
M	NA	4	33	15–49	37	14–85
M	MP	3	21	17–25	165	22–238
Imitation Mozzarella cheese						
Low moisture						
K	RC	3	25	15–31	20	12–26
M	MP	4	61	26–113	37	8.0–83
M	RC	2	32	23–42	12	9–14
Traditional						
K	CA	1	. . .	147	. . .	12
M	CA	2	315	308–329	11	10–12

[1]Means of duplicate analyses.

[2]K = Samples of known origin; M = samples collected at the market.

[3]NA = No addition, RC = rennet casein, CA = caseinate, and MP = milk protein. For the market samples, the declaration of the label is reported.

[4]The highest value of LAL and the lowest value of FUR were found in the same sample.

TABLE 6. Lysinoalanine (LAL) and furosine (FUR) in market samples of natural or processed Cheddar cheese.[1]

Type of product	n	LAL		FUR	
		(ppm in protein)		(mg/100 g of protein)	
		X̄	Range	X̄	Range
Natural	2	0.5	0.4–0.5	9.4	6.7–13
Processed	3	91	42–187	400	209–635

[1]Means of duplicate analyses.

In several countries, Cheddar cheese is used alone or together with Mozzarella cheese in preparation of pizza and related food (19). As expected, because of the mild thermal treatments occurring in cheese making (20), the LAL contents of natural Cheddar cheese and related processed products (Table 6) were comparable with those of natural Mozzarella cheese and imitation products, respectively. These results indicate that the presence of natural Cheddar cheese does not affect the analytical distinction that has been proposed in this article.

CONCLUSIONS

A precise quantification of the crosslinked AA, LAL, in the range of 0.4 to 20 ppm in protein allowed a highly significant ($P < 0.001$) distinction between natural Mozzarella cheese and dairy-based substitutes. Indeed, both the ingredients and thermal processing that have been adopted in the manufacturing of the imitation products led to LAL contents that were several times those occurring in Mozzarella cheese. The analytical method is suitable for routine application; excluding hydrolysis time, the entire analysis takes about 2 h, including HPLC. Furthermore, when the automatic procedure for SPE was used, the real working time was <30 min per sample.

Quality parameters should be incorporated into end use quality standards of Mozzarella cheese (24). The LAL index may be one of these parameters, and its application is advisable to meet choice and quality expectations of the consumer.
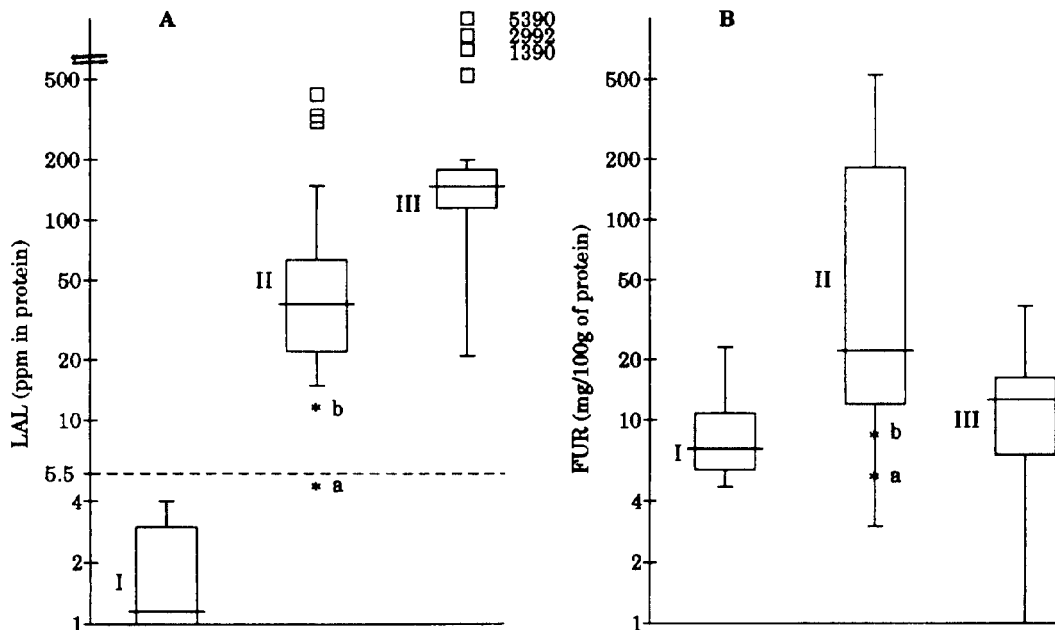

Figure 6. Box whisker plots of lysinoalanine (LAL) (A) and furosine (FUR) (B) values in all tested samples of natural Mozzarella cheese (I; n = 30), processed cheese and imitation Mozzarella cheese (II; n = 29), and rennet casein and caseinate (III; n = 15). The small boxes are the outliers of the plot, and the numbers are the LAL concentrations out of the scale. The broken line indicates the upper limit at the 3x standarad deviation confidence level of LAL content of natural Mozzarella cheese, and an asterisk indicates the blended samples of natural Mozzarella containing 10% (a) or 25% (b) (wt/wt) of imitation Mozzarella cheese with 24 ppm of LAL and 16 mg of FUR.

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