Research Article

A GC-FID method for analysis of Lysinoalanine

Antonia Montilla, José Ángel Gómez-Ruiz, Agustín Olano and María Dolores del Castillo

Instituto de Fermentaciones Industriales, Consejo Superior de Investigaciones Científicas, Madrid, Spain

Lysinoalanine (LAL) is an unwanted byproduct, which is formed during the processing of protein and protein-containing foods and feeds. A GC method for the quantitative analysis of LAL under conventional chromatographic conditions has been developed. The method was applied to the analysis of pure standard substances, boiled eggs, commercial caseinates, fresh cheese, fresh cheese made from milk supplemented with caseinate, and fresh cheeses adulterated with caseinate after cheese making process. Results demonstrated the reliability of the GC capillary chromatography for the analysis of LAL in protein containing foods. LOD and LOQ of 50 and 152 ppm of LAL in protein, respectively, were achieved. Range of linearity, precision, and accuracy of the method, measured using diaminopimelic acid as internal standard, were satisfactory for quantification purpose. The method might also be suitable for the quantitative analysis of other amino acids such as lysine and arginine. Results also indicated the utility of this methodology for detecting protein quality of egg products and caseinates as well as fresh cheese adulterations.

Keywords: Gas chromatography / Lysinoalanine / *N*(*O*)-*tert*-butyldimethylsilyl derivatives

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1 Introduction

Lysinoalanine [LAL, N^{ε}-(*R*,*S*-2-amino-2-carboxyethyl)-*S*-lysine] has been used as a marker of thermal damage in foods. The mechanism of formation of this crosslinking amino acid takes place in two main steps. The first step consisting in the formation of dehydroalanine residue from the β-elimination of cystine, serine, or its derivatives (*O*-phosphorylserine and *O*-glycosylserine) which is an hydroxide ion-catalyzed reaction, followed by a second reaction involving the double bond of dehydroalanine with a nucleophilic side chain of another amino acid, such as the ε-amino group of lysine [1, 2]. Formation of LAL along the protein chain may affect the nutritional and biological properties of the treated proteins. Decreasing of essential amino acids and reducing of protein digestibility, protein quality, mineral bioavailability, and utilization, within other nutritional and toxicological effects, are some of the negative consequences derived from LAL generation [1, 2]. High pH, temperatures, and long exposure time make these transformations favorable. Thermal treatments at basic pH are quite common in the food industry. Therefore, LAL may be found in widely consumed foods such as, baby food, cereal products, chicken meat, egg products, gelatine, infant formulas, meat products, caseinate, soy protein isolate, liquid milk, powdered milk and cheese among others [1, 3 – 16]. Significant concern arises with the detection of LAL in special foods, such as enteral nutrition formulas, where the quality of the protein used in their formulation is particularly relevant because these foods may be the sole nutrient source for patients during long periods of time [17]. In addition, LAL has been considered a better quality marker than Maillard reaction products for assessing the nutritional quality of proteins which are widely employed as an ingredient in the food industry, for instance caseins [9]. Therefore, strategies to minimize LAL formation during food processing and easy methods for its routine determination need to be developed.

Various analytical procedures have been evaluated for determining LAL in protein and protein-containing foods and feeds. Because LAL survives the acidic conditions of protein hydrolysis commonly used for the analysis of amino acids [1], its determination can be performed by ion-exchange chromatography (amino acid analyzer) with colorimetric and fluorometry detections after an acid protein digestion [18, 19], GC analysis of *n*-butyl esters of *N*(*O*)-trifluoroacetyl derivatives [6, 20, 21], GC-MS analysis of diasteromeric *N*(*O,S*)-perfluoropropionyl isopropyl esters [22]

Correspondence: Dr. María Dolores del Castillo, Instituto de Fermentaciones Industriales, Consejo Superior de Investigaciones Científicas, c/Juan de la Cierva 3, 28006 Madrid, Spain
E-mail: delcastillo@ifi.csic.es
Fax: +34-91-5644853

Abbreviations: DPA, diaminopimelic acid; **FID,** flame ionization detector; **LAL,** lysinoalanine; ***t*BDMSi,** *N*-(*O*)-*tert*-butyldimethylsilyl

or TLC and HPLC analysis [14, 23–26]. Most of the data recently published related to LAL analysis in foods have been acquired by the HPLC method proposed by Pellegrino *et al.* [9], based on derivatization with FMOC-Cl, SPE, RP chromatography, and fluorescence detection. The method is very sensitive (0.5–1 μg/g protein equivalent to ppm in protein); however, the sample preparation is complex and time consuming. The aim of this investigation was to find out a feasible procedure to achieve an easy and specific determination of LAL in the range of concentration commonly detected in protein containing foods. A GC-flame ionization detector (FID) method has been developed to achieve this purpose based on previous studies related to LAL analysis by GC [6, 20] and glycated and ascorbylated proteins by GC-MS [27].

2 Materials and methods

2.1 Chemicals

All chemicals used were of analytical grade. LAL was obtained from Bachem AG (Bubendorf, Switzerland). BSA, DL-2,6-diaminoheptanedioic acid also called diaminopimelic acid (DPA) and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoracetamide (MTBSTFA) were from Sigma (St. Louis, MO, USA). Hydrochloric acid was supplied by Panreac (Barcelona, Spain). Triethylamine (TEA) and DMF were from Merck (Darmstadt, Germany). High purity water was produced in-house using a Milli-Q Synthesis A10 System (Millipore, Bellerica, Mass., USA) and was used throughout.

2.2 Food samples

2.2.1 Boiled eggs

Grade A chicken eggs, medium size (53–63 g), laid in 7–8 days were obtained from a local farm and heated for 10, 20, 30, 45, and 60 min. Starting temperature was 20–25°C, after 10 min of heating 79–82°C were achieved and water boiling point arrived in 14 min. Shelling and separation of egg albumen from yolk were done carefully and manually. Samples were stored at −20°C until analysis. Treatments were carried out in triplicate.

2.2.2 Caseinates

Sodium ($n = 2$) and calcium caseinates ($n = 3$) were supplied by three European dairy companies.

2.2.3 Fresh cheeses

Raw cow's milk from a local farmer was used for cheese making. Previous to the manufacture of the cheeses, the raw milk was pasteurized at 65°C for 30 min. Three different batches were prepared in triplicate. A first batch was prepared without calcium caseinate (A, control). The second batch (B) was based on fresh cheeses from pasteurized milk supplemented with calcium caseinate to reach a final concentration of 15 g/L in milk. Briefly, after warming up the milk to 33°C, a lactic starter was added and the milk stirred for 35 min to help the acid development. Subsequently, 0.025 g/L calcium chloride was added and the milk stirred for another 5 min. Finally, 0.05 g/L of rennet was added. After 60 min of coagulation, the curd was cut and the whey drained from the cheese. This step was accompanied with heating the curd at 38°C for 20 min and gentle stirring followed by a first centrifugation step at $1700 \times g$, 23°C for 60 min. The purpose of these stages of the process was to increase the rate at which the curd contracts and squeezes out the whey making a hard curd. Once the curds had sufficiently hardened, salting for 30 min was performed. A second centrifugation step, as described above, was carried out. Then, cheeses were cut, stored in the fridge overnight, vacuum packed, and frozen at −20°C until analysis.

A third batch of cheese samples, consisting of fresh cheeses made as described above (A) to which 2.5, 5, 10, and 15% of commercial calcium caseinate containing 605.0 ppm of LAL in protein was added (C1, C2, C3, and C4), was also studied in the present paper. Adulterated cheeses were stored at −20°C until analysis.

2.3 GC analysis

2.3.1 Acid hydrolysis

Samples containing 40–50 mg of protein and DPA (160 μg) as an internal standard (IS) were thermally digested by 8 mL of 6 M HCl in Pyrex glass bottles at 110°C for 23 h. Prior incubation samples were degassed using a stream of helium for 2 min. The hydrolyzed samples were cooled at room temperature and filtered through a Whatman 40 paper filter.

2.3.2 Derivatization

Aliquots of filtrated hydrolyzates (0.5 mL) were evaporated to dryness at 38–40°C with a vacuum rotary evaporator. After evaporation, derivatization of the dried samples was carried out according to the methods of Hasenkopf *et al.* [27] and Woo and Chang [28]. DMF (165 μL) and TEA (15 μL) were added to the dry sample and stirred for 1 min. Afterwards, 100 μL of MTBSTFA, silylating reagent, were added and the reaction mixture was heated at 70°C for 60 min. Derivatization reaction was stopped by cooling to room temperature and the samples containing *N*-(*O*)-*tert*-butyldimethylsilyl (*t*BDMSi) derivatives of the amino acids were injected onto the GC column.

2.3.3 GC-FID analysis

The chromatography was performed by using a Hewlett-Packard HP6890 (Waldbronn, Germany) gas chromatograph equipped with an FID. A CP-SIL 5CB commercial fused-silica capillary column (100% bonded dimethylsiloxane, 25 m × 0.25 mm id, 0.25 μm film thickness; Chrom-

pack, Middelburg, The Netherlands) was used. The carrier gas (nitrogen) flow-rate was 1.2 mL/min. The make up gas was also nitrogen at the flow-rate of 20 mL/min. Injector and detector temperatures were 280 and 300°C, respectively. For analysis, 3 µL of *t*BDMSi amino acid derivatives was injected in splitless mode. The oven temperature was programmed at 100°C, held for 1 min, ramp to 250°C at 30°C/min, held for 42 min, ramp to 300°C at 50°C/min and held for 5 min. Data were acquired by means of HP ChemStations (Agilent Technologies, Wilmington, USA).

The range of linearity of FID response was checked by using a calibration curve of LAL. The calibration curve was constructed by adding different amounts of LAL to a standard mixture containing known quantities of BSA and DPA to obtain final concentrations of LAL from 152 to 3800 ppm in protein. The mixtures were hydrolyzed, derivatized, and the derivatives injected on to the column.

*t*BDMSi LAL derivative was quantified by internal standard method. All the analyses were carried out in duplicate and the data were the mean values expressed as mg LAL/kg protein (ppm in protein). Total nitrogen was determined by means of Kjeldahl method (International Dairy Federation, IDF Norma no. 24, 1964) [29] and the protein values were calculated using 6.25 and 6.38 as conversion factors for eggs and dairy products, respectively. LAL peak identification was achieved by migration time, standard addition, and MS analysis.

2.3.4 GC-MS analysis

An HP-6890 chromatograph coupled to an MD 5973 quadrupole mass detector (Hewlett-Packard, Palo Alto, CA, USA) was used. The separation of the *t*BDMSi amino acid derivatives was performed on a 30 m × 0.25 mm id × 0.25 µm film thickness, HP-5MS (bonded 5% phenyl, 95% dimethylpolysiloxane) fused-silica capillary column from Agilent Technologies. Helium was used as carrier gas. The oven temperature was programmed at 80°C, held for 1 min, ramp to 200°C at 50°C/min, ramp to 250°C at 10°C/min, held for 42 min, ramp to 300°C at 50°C/min and held for 5 min. Samples were injected in splitless mode. Mass spectrometer was operated in EI mode at 70 eV. Mass spectra were acquired using a G1701CA ChemStation Software (Hewlett-Packard).

2.3.5 Statistical analysis

Statistical analysis (Microsoft Excel 2000) of data was performed by one factor analysis of variance with a significance level of 95%.

3 Results and discussion

3.1 GC setup

Figure 1 shows the chromatogram of *t*BDMSi amino acid derivatives of hydrolyzed BSA spiked with pure LAL to a final concentration of 3800 ppm in protein, which is split into two zones to provide a better look of the GC-FID profile. *t*BDMSi-DPA eluted at 23.03 min while *t*BDMSi-LAL showed a retention time of 48.61 min corresponding to a relative retention time of 2.11. *t*BDMSi-LAL peak was not detected in untreated/nonspiked BSA hydrolyzates. Results indicated that DPA is a suitable internal standard for the determination of LAL by GC-FID agreeing with data previously described by Hasegawa *et al.* [6]. Purification of the sample was not necessary and a stable baseline during the analysis was obtained.

As it can be observed in Figs. 1A and B, both *t*BDMSi-DPA and *t*BDMSi-LAL were very well separated from the derivatives of the common 20 amino acids of proteins such as *t*BDMSi-lysine (relative retention time of 0.43) and *t*BDMSi-arginine (relative retention time of 0.36). Most of the 20 amino acids found in proteins eluted within 20 min of analysis with the exception of cystine, which showed a retention time of 47.2 min equivalent to a relative retention time of 2.05. Since chromatographic interferences have not been observed, simultaneous analysis of arginine, lysine, and LAL might be carried out under the proposed conditions.

*t*BDMSi-LAL was also analyzed by GC-MS. For this compound, one peak appeared in the chromatogram corresponding to a derivatization product with four silyl groups. Chemical structure and mass spectrum of *t*BDMSi-LAL are shown in Fig. 2. The most common MS fragments found in EI mass spectrum of the *t*BDMSi derivative of LAL agreed with those previously described by Hasenkopf *et al.* [27] for other *t*BDMSi derivatives of amino acids. The $[M^+]$ ion was not identified. Instead $[M\text{-}57]^+$ ion, which corresponds to the LAL with four silyl groups and the loss of $C(CH_3)_3$ was detected. Ion with *m/z* 387 $[M\text{-}159\text{-}131\text{-}15]^+$ due to a simultaneous loss of COO*t*BDMS, O*t*BDMS, and CH_3 can also be observed in Fig. 2 as the most prominent fragment.

LOD and LOQ were determined as three- and ten-fold the S/N near the retention time of LAL, respectively. LOD and LOQ of 50 ppm in protein and 152 ppm in protein, respectively, were obtained. Sensitivity for the determination was lower than that reported by HPLC (≈ 1 ppm in protein) [9] and similar to those reported using GC-NPD (nitrogen–phosphorus detection) [20] and ion-exchange chromatography (≥ 100 ppm in protein) [30]. The results suggested that the method provides the necessary selectivity avoiding a previous clean up step of the sample. In widely consumed foods such as pasteurized, UHT, and sterilized milks LAL values ranging from 17 to 69, 49 to 186, and 224 to 653 ppm in protein have been measured [14]. Upper limits of 200 ± 100 and 1000 ppm of LAL in protein for dried and liquid products, respectively, have been legally established in Germany for special foods like infant products [14, 31] and also recommended by the European regulatory organization [32]. Moreover, a low LAL content in foodstuffs (< 500 ppm in protein) has been recommended in order to

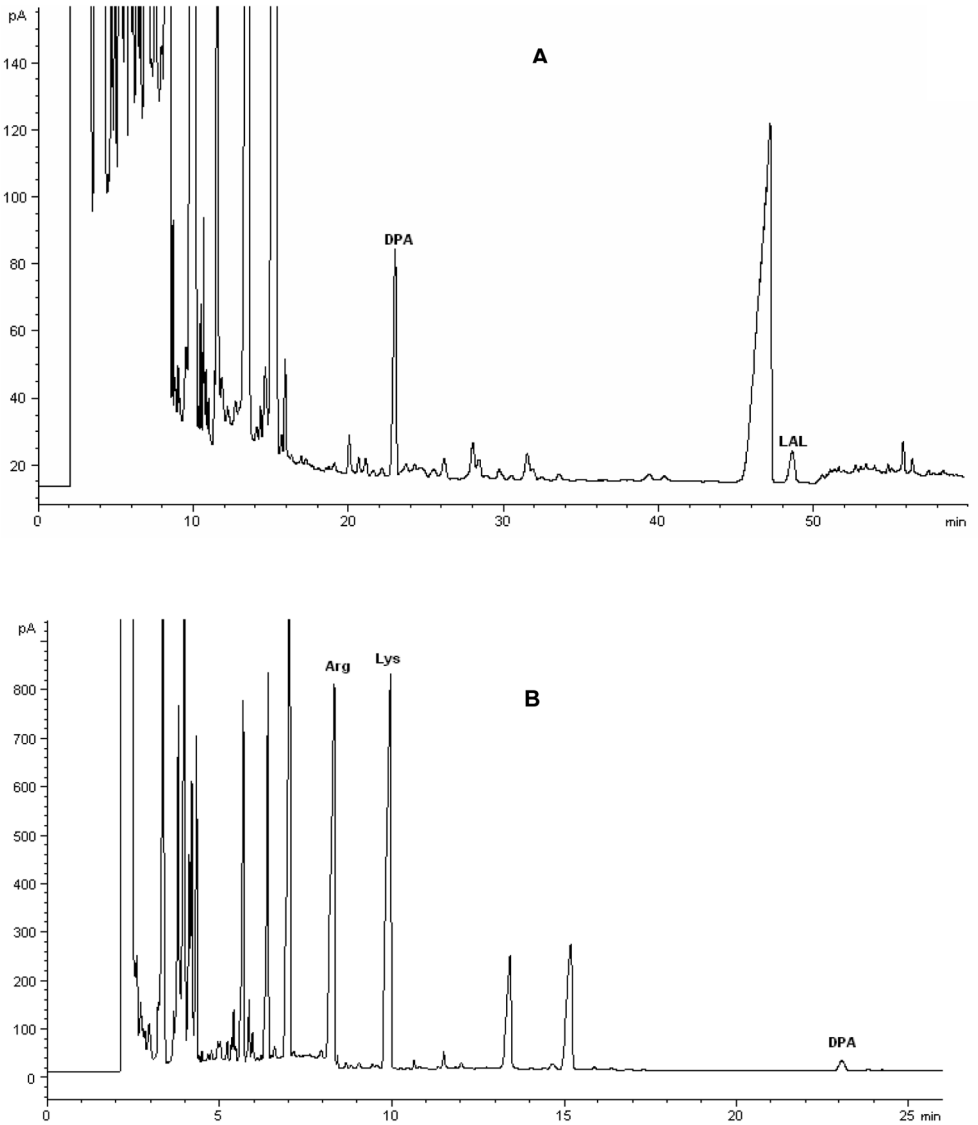

Figure 1. GC chromatogram of *t*BDMSi amino acid derivatives of hydrolyzed BSA spiked with pure LAL. GC profile is split into two zones as follows: Panel A shows those peaks corresponding to the *t*BDMSi derivatives DPA (DL-2,6-diaminoheptanedioic acid) (internal standard) and LAL, (lysinoalanine) while in Panel B can be observed the derivatives of arginine, lysine, and DPA.

obtain positive benefit from the nutritional point of view (Annual Report 2004. ALP Liebefeld – Posieux. Agroscope Liebefeld-Posieux (ALP). Swiss Federal Research Station for Animal Production and Dairy Products (www.alp.admin.ch/en/publikationen/docs/annual_report_2004.pdf, p. 21) amounts which are measurable by the proposed method. Most of the harmful effects associated to LAL have been caused by daily ingestion of doses higher than 500 ppm in protein [1].

Figure 3 shows an LAL standard curve ranging from 152 to 3800 ppm in protein. A linear relationship ($y = 3.6624x + 0.0155$) between the response measured as peak area and concentration of LAL over this range was observed. The regression coefficient of the curve was 0.9859. One factor analysis of variance of the response factor of LAL demonstrated significant homogeneity of the variance between the samples over the range of concentration studied since an F-value of 0.7327 with $p = 0.8235$ ($p > 0.05$) was obtained. Data agreed with those reported by Hasegawa *et al.* [6]. The precision of the method was estimated by the analysis of BSA sample spiked with 380 ppm of LAL in protein within the same day (repeatability, $n = 3$) and in different days (intermediate precision, $n = 4$), obtaining RSDs of 5.23 and 7.75, respectively. Recovery of the method was also evaluated by adding known amounts of LAL to pure BSA, raw albumen, and fresh cheese (A).

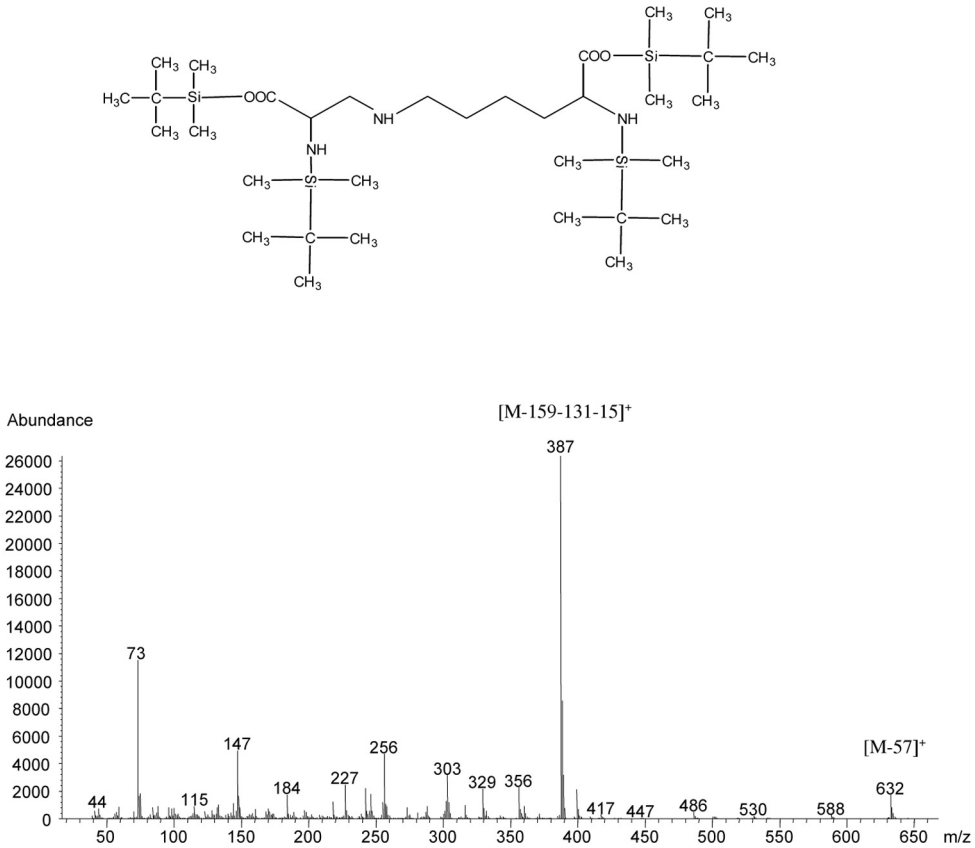

Figure 2. Chemical structure and mass spectrum of *t*BDMSi derivative of pure LAL obtained by electron impact ionization GC-MS.

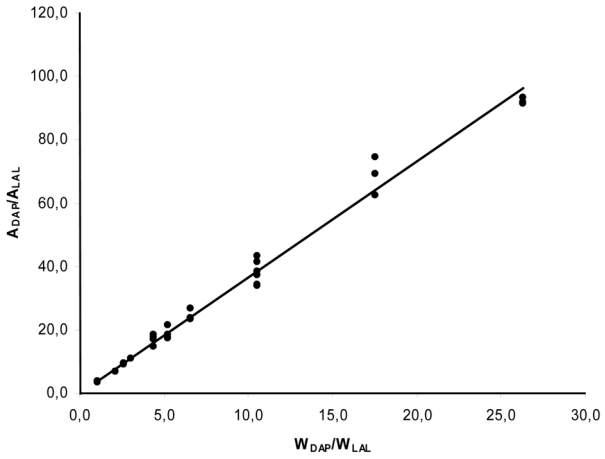

Figure 3. Relationship between response (Areas) ratio and weight ratio of *t*BDMSi-LAL and *t*BDMSi-DPA obtained by capillary GC-FID analysis.

Average values of 94.0, 90.2, and 91.1% of LAL were recovered from hydrolyzates of BSA, raw albumen, and fresh cheeses, respectively.

Although the GC analysis of *t*BDMSi-LAL has been previously proposed, this is the first time that capillary columns commercially available are used. The use of commercial GC columns could facilitate the development of standardized procedures. The method here reported offers significant improvements with respect to previous methods based on GC analysis of *t*BDMSi-LAL in terms of simplicity, time of analysis, and resolution. In addition, it allows simultaneous quantitative determination of LAL and the common 20 amino acids found in proteins, among them lysine which is an essential amino acid considered as a chemical indicator for the control of protein nutritional quality in processed foods.

3.2 Analysis of LAL in food samples

To study the applicability of the validated method, the presence or formation of LAL in different food matrices (boiled eggs, caseinates, and fresh cheeses) was evaluated.

3.2.1 Crosslinking during boiling of eggs

Table 1 shows the concentration of LAL found in fresh and boiled albumen and yolk. No LAL was observed in raw samples. LAL content increased progressively as a function of the heating treatment time. Rate of LAL formation in boiled white was faster than that detected in the boiled yolk egg. Similar levels of LAL in both, albumen and yolk, have been found by Hasegawa *et al.* [6].

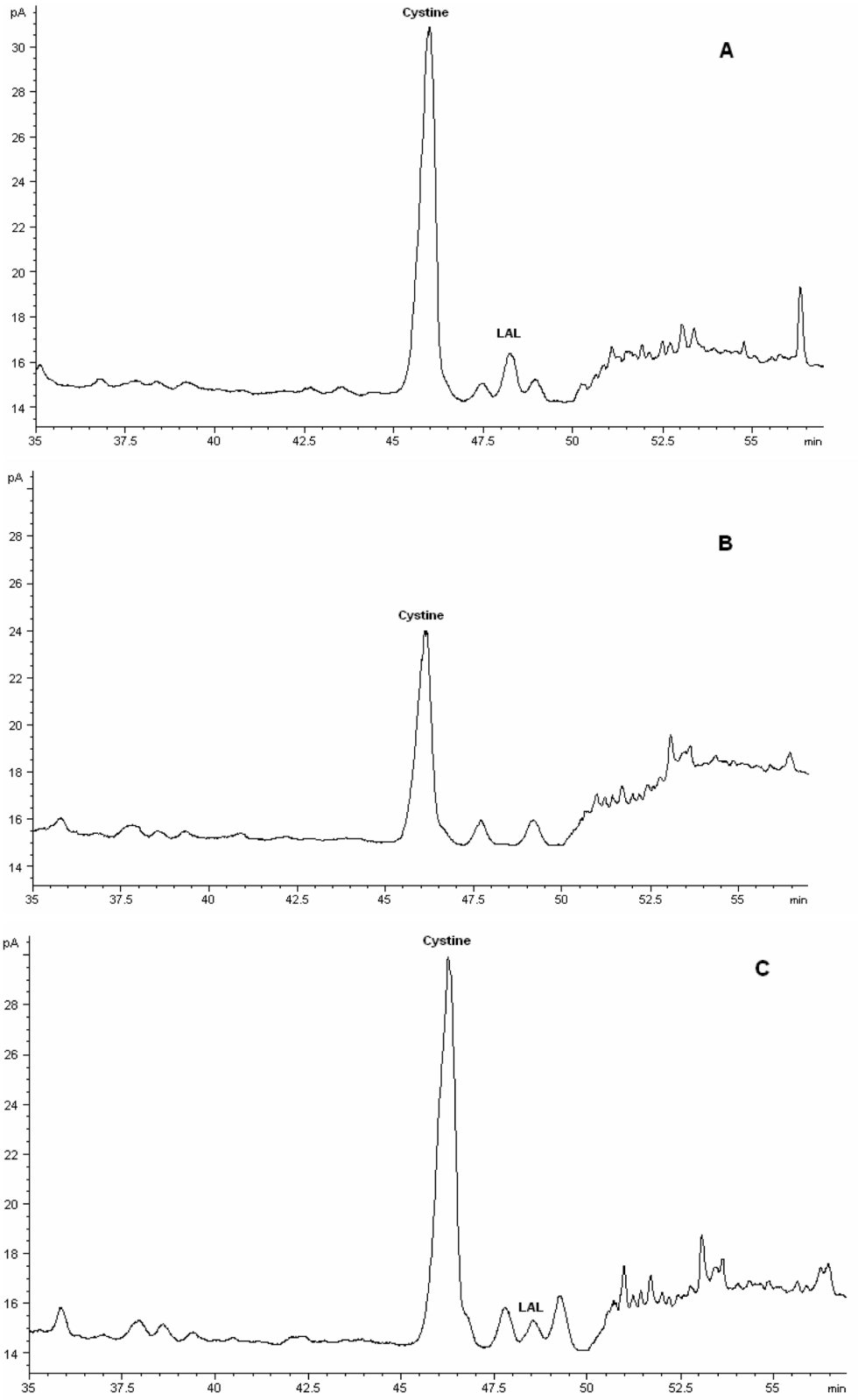

Figure 4. GC-FID profiles of *t*BDMSi amino acid derivatives of hydrolyzed commercial calcium caseinate (A), fresh cheese (B), and fresh cheese made from milk containing 15 g/L of calcium caseinate (C).

Table 1. LAL contents formed by boiling eggs

Heating conditions	LAL content (ppm in protein)	
	Egg albumen	Egg yolk
Raw	ND	ND
79 – 82°C, 10 min	Traces	ND
Heating 20 min (6 min boiling)	153.3 ± 26.6	ND
Heating 30 min (16 min boiling)	211.0 ± 25.4	Traces
Heating 45 min (31 min boiling)	622.4 ± 58.7	Traces
Heating 60 min (46 min boiling)	677.0 ± 63.4	154.4 ± 28.5

Results are expressed as mean ± SD ($n = 3$). ND = no detected.

3.2.2 LAL in commercial caseinates and experimental fresh cheeses

LAL values up to 605.0 ppm in protein were detected in commercial caseinates. Data agreed with those previously reported by other authors [1, 10]. GC profile corresponding to calcium caseinate, which showed the highest LAL content within the samples under study, is presented in Fig. 4A.

Table 2 shows the LAL content of both fresh cheeses and fresh cheeses made from milk supplemented with calcium caseinate. Chromatograms of both cheeses are shown in Figs. 4B and C. No LAL was detected in fresh cheeses (Fig. 4B and Table 2). No LAL or quantities of this compound lower than 69 ppm have been reported in pasteurized milk [1, 14]. LAL is not a suitable marker for milk but it is for caseins and caseinates [10]. In cheeses based on milk supplemented with 15 g/L of calcium caseinate (B), values from 140.2 to 207.6 ppm of LAL in protein were detected (Table 2). Differences in LAL contents might be due to a differential rate of caseinate absorption during cheese making which is in agreement with protein data. Higher values of LAL corresponded to higher protein contents (Table 2).

Adulteration of fresh cheeses with amounts of caseinates ranging from 2.5 to 15% (C) was detected by using the GC methodology described here. A good relationship between the expected and the experimental LAL data was obtained. Calculated values for LAL in fresh cheeses adulterated with 10% (167.1 ppm in protein) and 15% (208.8 ppm in protein) of caseinate agreed with those expected (179.8 and 234.4 ppm in protein, respectively) suggesting the feasibility of the method to detect cheese adulterations with caseinates like Mozzarella substitutes (1, 9 – 10, 16).

Table 2. Protein and LAL contents of experimental fresh cheeses

Sample	LAL (ppm in protein)	Protein (g/100 g of product)
A1	0	20.9
A2	0	21.6
A3	0	21.1
B1	158.9 ± 26.5	22.3
B2	207.6 ± 18.6	23.1
B3	140.2 ± 9.5	21.3

A, control fresh cheeses and B, fresh cheeses made from milk supplemented with 15 g/L of caseinate. Results are expressed as mean ± SD ($n = 3$).

4 Concluding remarks

The proposed procedure allows the quantitative analysis of LAL by gas capillary chromatography using conventional conditions without complex and time-consuming preparations. In addition, lysine, arginine, and other amino acids may be analyzed by employing the proposed methodology. Results indicate the feasibility of this analytical tool for detecting protein quality of egg products, caseinates, and fresh cheese due to adulterations.

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