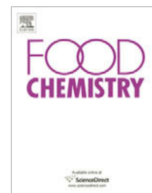
Food Chemistry 116 (2009) 799–805

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Analytical Methods

Quantitation of lysinoalanine in dairy products by liquid chromatography–mass spectrometry with selective ion monitoring

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ARTICLE INFO

Article history:
Received 25 September 2008
Received in revised form 5 March 2009
Accepted 8 March 2009

Keywords:
Lysinoalanine
Liquid chromatography–electrospray mass spectrometry
Selected ion monitoring
Milk powder
Calcium caseinate

ABSTRACT

The unnatural amino acid lysinoalanine (LAL) has been identified in milk and cheese products by liquid chromatography mass spectrometry (LC/ESI/MS) with selective ion monitoring (SIM) of the 9-fluorenyl-methylchloro-formate (FMOC) derivative. LAL is not present in raw milk or derived from Mozzarella cheese; however, high amounts of LAL are found in calcium caseinate and milk powder. As expected, milk fortified with caseinate or whey protein powder produces cheese with higher LAL content. Our analytical procedure is based on the simultaneous detection of specific ion masses of the FMOC–LAL derivative and the *N*-ε-methyl-lysine internal standard. A linear relationship was observed within the 0.2–20 ppm concentration range, in addition to a high correlation coefficient and ~3% relative standard deviation.
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1. Introduction

European regulation allows for the use of milk powders in mass-produced cheeses, while incorporation of cheaper milk powders into protected denomination of origin (PDO) cheeses is prohibited. To detect and quantify key compounds in highly heated liquid milk and milk powders, much research has focused on phosphorylated serine and threonine residues that are heat-susceptible and known to yield dehydroalanine (DHA) or methyldehydroalanine (Me-DHA), respectively (Hurrell, Carpenter, Sinclair, Otterburn, & Asquith, 1976). Reaction of the conjugated carbon–carbon double bond with a nucleophilic amino (Lys, His) or thiol (Cys) group produces lysinoalanine (LAL), histidinoalanine (HAL) and lanthionine (LAN) residue-containing proteins, respectively (Cuq & Cheftel, 1985; Finot, 1983; Friedman, 1999). HAL was first found in heated milk by (Henle, Walter, & Klostermeyer, 1993). The same research group reported the formation of lysinomethylalanine (LMeAL) and histidinomethyalanine (HMeAL) in milk products (Walter, Henle, & Klostermeyer, 1994). High molecular weight aggregates consequently form through bifunctional protein crosslinks (Erbersdobler & Holstein, 1980; Dehn-Muler, Muller, & Erbersdobler, 1991). Current research efforts have been motivated by the need to develop specific, rapid and sensitive analytical procedures for evaluation of unnatural amino acids. LAL indicates the presence of casein and caseinate powder in cheeses. *Pasta filata* and imitation processed cheeses are differentiated by LAL levels (Faist, Drusch, Kiesner, Elmadfa, & Erbersdobler, 2000). HPLC-based methods separating fluorescent 9-fluorenyl-methylchloro-formate (FMOC) (Pellegrino, Resmini, De Noni, & Casotti, 1996) or dansyl (DNS)–LAL derivatives (Faist et al., 2000; Badoud & Pratz, 1984) are presently used as tools for monitoring LAL levels in dairy products. Using a DNS–Cl reagent, LAL content in raw and pasteurised milk was found to be in the range of 4–24 or 17–69 mg kg^{-1} crude protein, respectively. UHT-treated milk and sterilized milk displayed higher LAL levels of 186 and 653 mg kg^{-1} crude protein, respectively (Pellegrino et al., 1996). However, accurate evaluation of low LAL levels can be hindered by the presence of free amino acids and/or other structured co-eluting molecules.

Methods that combine HPLC and MS are known to provide structural information and reliable quantitative data. Notwithstanding, a limited number of studies using MS have detected crosslinked proteins and peptides (Bunk & Welch, 1997; Sannolo, Mamone, Ferranti, Basile, & Malorni, 1999). To date, liquid chromatography (LC) on-line coupled to Electrospray Ionisation (ESI)/MS has been extensively used to obtain absolute quantification of

Abbreviations: DHA, dehydroalanine; DNS–LAL, dansyl chloride; FMOC, 9-fluorenyl-methylchloro-formate; HAL, histidinoalanine; LAL, lysinoalanine; LAN, lanthionine; LC/ESI/MS, liquid chromatography–electrospray mass spectrometry; Me-DHA, methyl-dehydroalanine; Me-DHA, methyl-dehydroalanine; *N*-ε-Me-Lys, *N*-ε-methyl-lysine; PDO, protected denomination of origin; SIM, selective ion monitoring.

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doi:10.1016/j.foodchem.2009.03.041

small organic compounds (Kautiainen, Fred, Rydberg, & Tornqvist, 2000; Koc, Mar, Ranasinghe, Swenberg, & Zeisel, 2002).

LC–ESI/MS with single ion monitoring (SIM) detection is expected to quantify amino acids. Here, we used direct LC–ESI/MS analysis in positive SIM of the FMOC–LAL derivative without sample enrichment. In this sense, the use of LC–MS technique represents an advance in comparison with traditional HPLC method (Pellegrino et al., 1996) since the accurate molecular weigh measurement provides a further discriminating parameter for detection and quantification, thus avoiding most false positives due to interference of co-eluting compounds.

2. Experimental

All experiments were carried out in triplicate.

2.1. Chemicals and reagents

All chemicals were obtained from Sigma–Aldrich (Milan, Italy) unless otherwise specified. HPLC-grade solvent and hydrogen chloride were supplied from J.T. Baker (Philipsburg, NJ, USA). Synthetic LL- and DL–LAL was from Bachem (Bubendorf, Switzerland), and 9-fluorenylmethilchloroformate was from Pierce (Rockford, IL, USA).

2.2. Dairy samples

Samples of fresh milk and natural or imitation Mozzarella cheese were obtained from specialized factories. Cheese samples containing dairy powder were run separately in vats in a pilot plant starting with raw milk and adding known amounts of commercial calcium caseinate (0.06 to 0.5 kg/100 L milk) or milk protein powder (1.5 kg/100 l milk) (NZMP, Lecco, Italy). Two *pasta filata* cheese samples were obtained by stretching a properly acidified curd with a commercially processed cheese into 17.6% and 41.7% final proportions.

Cheese analysis: protein content was determined by Kjeldahl ($N \times 6.38$) (International Dairy Federation, 1993).

2.3. Preparation of samples for LAL analysis

Mozzarella cheese (250 mg) was mixed with 6 N HCl (8 ml) in a glass tube, flushed with nitrogen to eliminate air and sealed under vacuum pressure. For all milk samples, 2 mL aliquots were dried and treated as above. The resulting mixture was hydrolysed at 110 °C under constant temperature for 24 h, cooled to room temperature and filtered through a 0.45 μm PVDF filter (Millipore, Milan, Italy). One microgram of *N*-ε-methyl-lysine (*N*-ε-Me-Lys) was added to a 100 μl filtrate, and the resulting solution was dried in a Speed Vac centrifuge (Savant Instruments, USA). After a twofold addition of distilled water followed by vacuum drying, the pellet was dissolved in 200 μl of distilled water and 200 μl of 0.4 M sodium borate at pH 12. Then, 400 μl of freshly prepared FMOC reagent (5 mg/mL in acetonitrile, ACN) was added to the solution, and the reaction mixture was left to stand at room temperature for 60 min. The solution was diluted 1:2 (v/v) with 0.1% TFA, and a 100 μl aliquot of the derivatized sample was injected for LC/MS analysis. To analyse calcium caseinate samples, the same procedure as above was used starting with a weight of 60 mg. The *N*-ε-Me-Lys standard (1 μg) was added after acid hydrolysis for quantitation.

2.4. LC–ES/MS/SIM analysis

Separation by LC was performed using a C18 reversed-phase column (218TP52, 5 μm, 250 × 2.1 mm, Vydac, Hesperia, CA, USA) with a flow rate of 0.2 mL/min on a Hewlett-Packard Model 1100 system. Solvent A was 0.03% TFA in water, and solvent B was 0.02% TFA in ACN. Separation of FMOC-amino acids was carried out in a linear gradient of 30–100% Solvent B for 50 min. After sample injection, the column was washed for 5 min with starting buffer before entering the gradient. ESI/MS analysis was carried out on a platform mass spectrometer (Micromass, Manchester, UK). Mass spectra were scanned from 1000 to 200 Da at a scan cycle of 3 s per scan. The source temperature was 180 °C, and the cone voltage was held at 20–40 V depending on the experimental conditions. Mass scale calibration was carried out using myoglobin as a reference compound. Charged ions of FMOC–LAL derivatives (900.3) and *N*-ε-Me-Lys (605.3) were monitored using the SIM mode with the mass window for each channel fixed at 0.08*m*/*z*.

2.5. Calibration curve

The calibration curve was obtained by analysing samples prepared from 1 μg of *N*-ε-Me-Lys mixed with 15.6–500 ng LAL using either water or dried raw milk hydrolysate as diluent reconstituted to the initial volume. Samples were derivatized and analysed by LC–ESI/MS/SIM as described above. Each standard concentration was run from lowest to highest in triplicate. The HPLC peak was analysed at a low cone voltage (20 V) to suppress partial FMOC–LAL fragmentation.

2.6. Precision and accuracy

Relative standard deviation (RSD, %) and accuracy as percentage of relative error (RE, %) were determined from preliminary experiments by spiking natural Mozzarella cheese with 1, 0.25, 0.0625 and 0.015 μg of LAL (QC samples). The QC samples were then analysed after standing at room temperature for 24 h and compared with freshly prepared samples. Significance of the results was determined by Student's *t*-tests ($p < 0.05$).

3. Results

3.1. Derivatization of LAL with FMOC and detection by LC–ESI/MS

The differential effects of using synthetic LAL, milk sample hydrolysate and *N*-ε-Me-Lys (internal standard) were evaluated through full-scan ESI/MS analysis using the positive-ion mode. FMOC reagent was directly added to the protein hydrolysate and injected without any prior workup on the HPLC column coupled on-line to ESI–MS (Fig. 1). While monitoring the column effluent for positively charged ions, a major signal at *m*/*z* of 605.3 was identified (with 32.9 min retention time (t_R)), consistent with the predicted mass of two FMOC-*N*-ε-Me-Lys derivatives (Fig. 1, inset A).

In contrast, the peak at t_R 35.3 min (*m*/*z* 900.3 Da) had a molecular mass value similar to that of three FMOC–LAL derivatives (expected $MH^+ = 900.3$) (Fig. 2, inset B).

The FMOC reagent is thus effective in converting both primary and secondary amino groups of LAL into FMOC derivatives.

The success of detecting trace amounts of LAL depends on many factors, among which sensitivity of diagnostic information would be maximised. A series of mass spectra was acquired at increasing values of activating cone voltage, and ion signal heights were monitored.

At 20 V, the FMOC–LAL molecular ions were the most intense (Fig. 2B), while a large increase in fragment yield (*m*/*z*678.3 and 456.2) was seen at 40 V, corresponding to the loss of one or two FMOC groups by FMOC–LAL molecular ions (Fig. 2A). The loss of the third FMOC group was obtained only at higher cone voltage values (data not shown). The FMOC-*N*-ε-Me-Lys derivative fragmented under similar mechanisms (Fig. 2C and D).

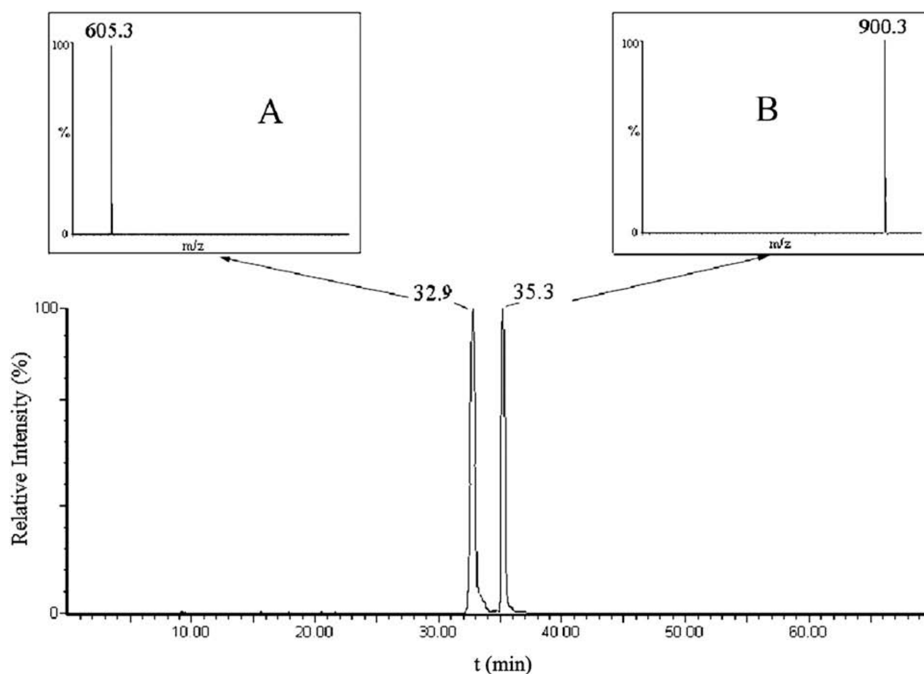

Fig. 1. TIC of a mixture of LAL and *N*-ε-Me-Lys after derivatization with the FMOC reagent. Full-scan ESI mass spectra were taken at 20 V cone voltage. Peaks eluted at 32.9 and 35.3 min corresponding to FMOC-*N*-ε-Me-Lys (inset A) and -LAL (inset B), respectively.

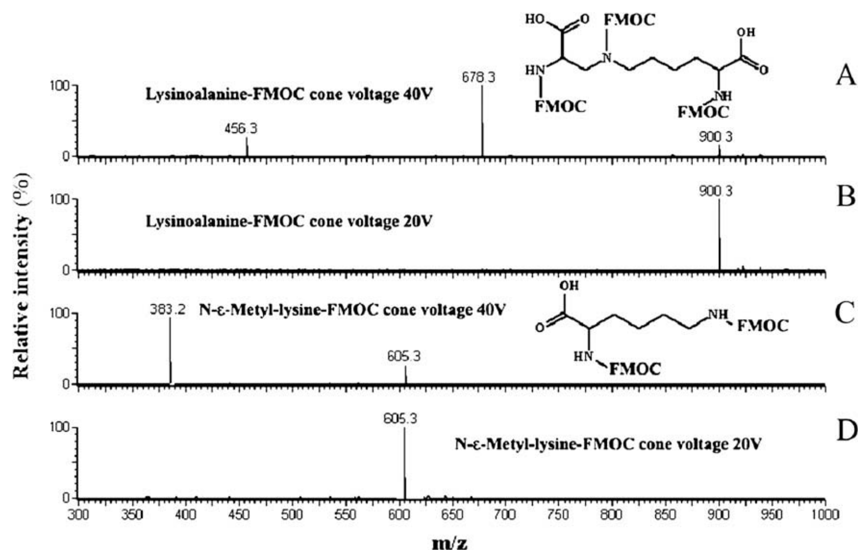

Fig. 2. Full-scan mass spectra of FMOC–LAL and FMOC-*N*-ε-Me-Lys at cone voltages of 40 V (A and C) and 20 V (B and D). Chemical structures are shown at the top of the spectra.

In order to enhance both the sensitivity and specificity of the response, mass selective detection along the TIC profile (Fig. 1) was used to provide highly specific chemical information including molecular mass and/or characteristic fragment ion(s) directly linked to the presence of LAL–FMOC. The results of mass selective extraction are shown in Fig. 3 for two molecular ions and their characteristic fragments, tuning the cone voltage to 20 and 40 V. At 40 V, signals were recorded at *m/z* 678.3 (Fig. 3B) and 456.2 (Fig. 3A) with the same t_R of parent ions at *m/z* 900.3 (Fig. 3C), thus confirming that both originated from the three FMOC–LAL derivatives. Using this procedure, a fragment at *m/z* 383.2 (Fig. 3D) was observed at the t_R of the two parent FMOC-*N*-ε-Me-Lys derivative ions at *m/z* 605.3 (Fig. 3E).

Therefore, both LAL and *N*-ε-Me-Lys were converted into their respective full FMOC derivatives in a quantitative manner and detected at the expected t_R values.

To ascertain whether possible isobaric compounds could interfere with LAL in both spiking and sample analysis, the tri-FMOC LAL derivative was analysed by LC–ESI/MS/SIM. Profiles of raw milk (Fig. 4B and D) and raw milk fortified with known amounts of *N*-ε-Me-Lys and LAL (Fig. 4A and C) are shown in Fig. 4. As expected, neither LAL nor *N*-ε-Me-Lys were detected in raw milk samples, indicating that the matrix did not influence LAL content of the samples. Coincidentally, the sample matrix did not appear to influence LAL analysis. No derivatized matrix compounds were found to interfere with chromatography of the analytes of interest

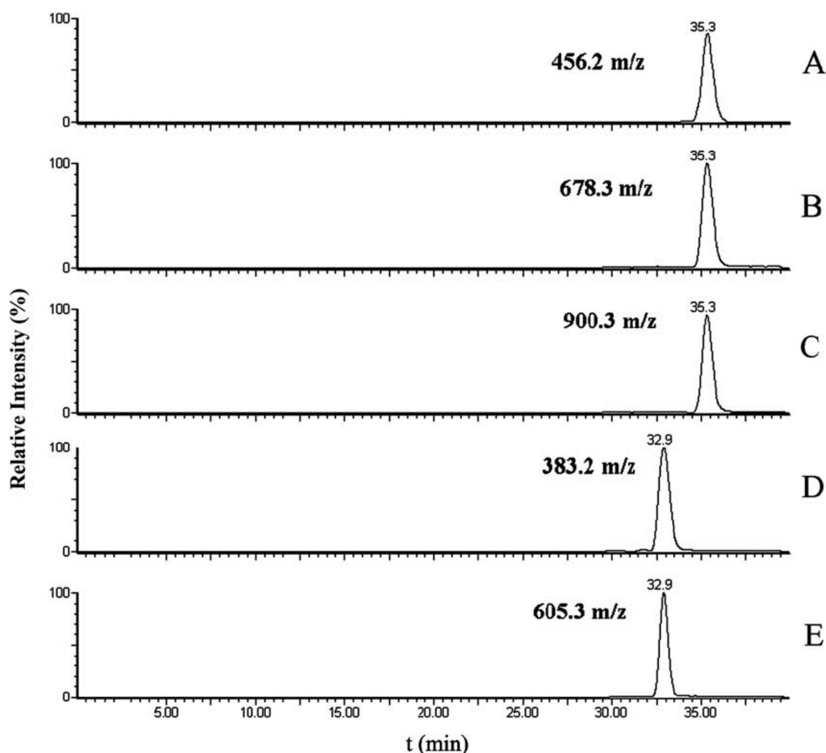

Fig. 3. LC–ESI/MS/SIM at 40 V and *m/z* 605.3 (E) or *m/z* 383.2 (D) for FMOC-*N*-ε-Me-Lys and *m/z* 900.3 (C), *m/z* 678.3 (B) or *m/z* 456.2 (A) for the FMOC–LAL derivative.

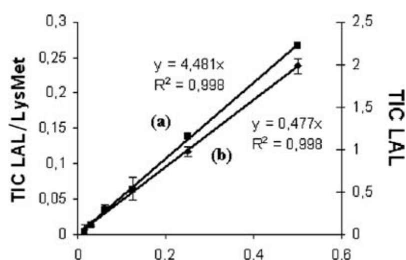

Fig. 4. Derived calibration curve for LAL quantification. Standard concentrations 409 ranged between 15.6 to 500 ng LAL/100 mL. The full range of the curve is shown in (A), while the same curve normalised with respect to the standard for the inclusion of 1000 ng of *N*-ε-Me-Lys is shown in (B).

containing signals at the expected t_R of LAL- and *N*-ε-Me-Lys-FMOC derivatives (data not shown). Thus, LAL standard solutions could be prepared by sequential dilutions from a stock solution with either water or reconstituted milk acid hydrolysate.

In addition, the use of the SIM procedure dramatically reduced the number of HPLC peaks, thereby increasing the signal-to-noise ratio. As the objective was the development of a method for quantification of LAL in dairy products (namely *pasta filata* cheeses), contribution of curd fusion followed by stretching to LAL formation was evaluated. At the expected t_R value, neither FMOC–LAL nor FMOC-*N*-ε-Me-Lys SIM signals were detected (data not shown), suggesting that the making of Mozzarella cheese did not significantly affect LAL amounts when raw milk was used.

Because the LAL level was critical, we arbitrarily fixed the threshold value for LAL at 0.2 ppm to qualify the cheese as genuine. This partially complies with the findings of Pellegrino et al. (1996) but a wider LAL range (0.4–4.0 ppm) is proposed to distinguish genuine Mozzarella cheese from processed cheese containing casein and caseinates. In order to detect low levels of LAL by SIM analysis, the acid hydrolysate of Mozzarella cheese samples was fortified with synthetic LAL.

Addition of LAL to the cheese hydrolysate did not effect the LAL amount or retention time, which was similar to that of authentic LAL.

As the upper LAL limit had not been fixed by regulatory directives, LAL quantification required the use of a standard curve for analysis of dairy samples. The peak area along the LC–ESI/MS/SIM pattern was measured to obtain a calibration curve using *N*-ε-Me-Lys as an internal standard.

3.2. Calibration curve

LAL and the internal standard were converted to FMOC-derivatives, analysed by SIM analysis, and the absolute area measured. The area value was plotted *vs.* the LAL concentration (Fig. 4). Calibration plots were obtained by analysis of solution samples containing increasing amounts of LAL in the presence or absence of a fixed amount of the internal standard.

Solvent used was either water or milk hydrolysate. In both cases, the SIM response was linear (r^2 value = 0.998) within the range of concentrations explored (15.6–500 ng LAL/100 μl injected) at various times in triplicate (Fig. 4). The results indicated satisfactory instrument repeatability, and the ion intensities of diluted LAL standard samples were not influenced by the presence of the internal standard. Calibration curves had no *y*-intercept, indicating that the internal standard did not contribute to the analyte ion abundance. Subsequent LAL measurements were then conducted on dairy samples exclusively in the explored concentration range.

3.3. Analytical precision, accuracy, reproducibility and recovery

Four assays were carried out on the same sample over two days to evaluate reproducibility. The value of the standard deviation was 0.44% intraday and increased for interday (48 h). Therefore, analysis runs must be made consecutively and completed within 24 h of the start.

The repeatability of the method was evaluated using a standard of 0.0625 μg LAL. Relative standard deviation of the peak area and retention time were less than 0.037% and 0.01%, respectively. Eight replicates ($n = 8$) at 0.015, 0.0625, 0.25 and 1 μg LAL were used to make the low- to high-range concentrations. The mean interday accuracy ranged from 103–110% for SIM detection with a mean CV < 6.4%. The mean intraday precision for all standards was <10% of the expected concentration, and recovery was about 110% in Mozzarella cheese spiked with known amounts of LAL.

3.4. Quantitative analysis of LAL in caseinate and cheese samples

Calcium caseinate powder and Mozzarella cheese hydrolysate samples were added to the internal standard and submitted for FMOC derivatization. The crude mixture was then directly analysed by LC–ESI/MS in the SIM mode. At the LAL- and *N*-ε-Me-Lys-FMOC derivative t_R, the *m/z* 900.3 and 605.3 signals were specifically measured by the software program, and LAL concentrations were calculated taking into account the relative areas and corresponding LAL values within the calibration plot using SIM analysis (Fig. 5).

To accurately compare and quantify FMOC–LAL, an internal standard was added to the samples prior to addition of the FMOC reagent. If endogenous LAL was converted into a tri-FMOC derivative, a similar and full conversion should occur with the internal standard (not showed). As LC–ESI/MS/SIM readily detects both FMOC–LAL derivatives, the stoichiometric conversion of LAL into FMOC derivatives was controlled by reading of expected signals in apposite channels (data not shown). The FMOC–LAL derivative peak was observed (Fig. 5C, arrow), whereas the signals acquired in the SIM mode at *m/z* 900.3 and *m/z* 605.3 for *N*-ε-Me-Lys are shown in Fig. 5A and B. The interpolated signals from the calibration curve allowed calculation of LAL levels in the samples and were consistent with previous LC results (data not shown), where LAL was analysed by HPLC (Pellegrino et al., 1996). Lower recoveries were attributed to anion resin use, producing ineffective adsorption of FMOC–LAL derivatives into the sorbent phase. The SIM procedure showed constant LAL levels beyond 100 ppm, irrespective of the origin of calcium caseinate in the sample. However, as problems in stability of DHA- and LAL-caseinate-containing powders could be contributed to erratic recoveries, both calcium caseinate and milk protein powder were incorporated into the liquid milk to produce Mozzarella cheese. No compounds in the two *pasta filata* cheese varieties were found to interfere with LAL by SIM analysis. Milk samples containing 0.06%, 0.5% and 1.5% calcium caseinate had 4.5, 33.5, and 70.9 ppm LAL (samples P6, P2, and P9), respectively (Table 1). Further comparison of the measured and expected LAL content was made on raw milk (MM), milk proteins (MP) and calcium caseinate (CC) powder. In all cases, the measured LAL level was consistent with that of the expected (Table 1). The lowest recoveries were observed for two composite *pasta filata* cheese samples (P20 and P21), which may be attributed to unequal incorporation of the processed cheese during curd stretching. Regardless, this procedure was useful in detecting processed

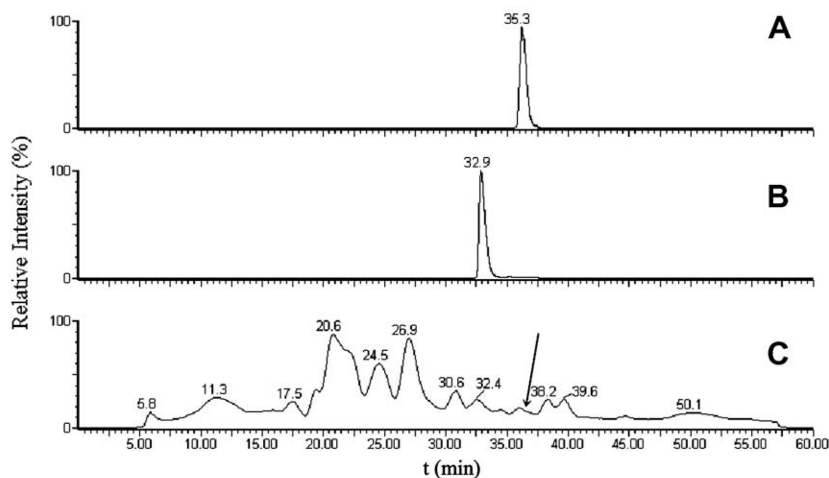

Fig. 5. Total ion current of a calcium caseinate sample analysed by LC–ESI/MS in the presence of an internal standard. Arrow in (C) identifies the FMOC–LAL derivative peak. Signals at *m/z* 900.3 for LAL (A) and *m/z* 605.3 for *N*-ε-Me- Lys (B) were acquired in a separate run using the SIM mode.

Table 1
Example of LAL evaluation by the LC–ESI/MS–SIM procedure in natural and adulterated milk and *pasta filata* cheese samples containing a known amount of calcium caseinate or process cheese. P, and C, samples of Mozzarella and Caciocavallo Silano cheese; PP, MM, and SS, samples of calcium caseinate, liquid milk, and milk proteins respectively; CC, MP, and PC, calcium caseinate, milk protein, and process cheese commercial preparation.

Sample	Ingredient for cheese	Dose per 100 kg raw milk	LAL measured (ppm)	LAL expected (ppm)
P2	CC[a]	0.5	33.5	32.2
P6	CC	0.06	4.5	4.5
PP	CC, powder		775.0	700.0[a]
MM	Pure raw milk		0	n.d.
SS	MP°, powder		70.9	–
P9	MP	1.5	70.9	69.1
P20	PC+	1.42	51.5	73.4
P21	PC	4.76	49.5	82.7
C1	CC	1.38	220.1	220.1
C3	CC	0.46	88.8	89.9
C5	MP	2.31	28.7	28.8

[a] Evaluation according to the Pellegrino et al procedure.

and *pasta filata* cheese mixtures (Table 1). Caciocavallo Silano PDO cheese samples belong to the *pasta filata* family and were found to have reduced moisture levels (∼40%) when prepared at stretching temperatures of >70 °C (C1, C3, and C5 samples). These cheeses gave expected results, further confirming that LAL could be traced in both heated and dried milk products.

It is also important to underline that the described methodology could be extended to various kinds of food which are subject to intense heat treatment, including bakery products (Bosch et al., 2007). Furthermore the recent detection of LAL in human cataractous lens suggests that future application could be applied to clinical determinations on physiological samples (Linetsky, Hill, LeGrand, & Hu, 2004).

4. Discussion

Preliminary assays for direct detection of crosslinked proteins by LC–ESI/MS were unsuccessful, as native protein signals likely obscured those of LAL-containing proteins occurring in trace amounts. Alternatively, LAL can be imprecisely released through a long and complex sequence of variable proteolysis (Henle, Walter, & Klostermeyer, 1994). Thus, there is an increasing interest in using acid hydrolysis of proteins to directly convert LAL (or other unnatural amino acids) to fluorescent FMOC or DNS derivatives (Faist et al.,2000; Badoud & Pratz, 1984). LAL has been detected in many dairy products in this manner either directly (Faist et al., 2000) or after a cleanup step prior to HPLC separation (Pellegrino et al., 1996) to remove any non-specific signals that compromise accurate quantification. However, the major weakness of this approach is the inability to obtain structural information for relevant LAL derivatives.

In contrast, MS reduces the possibility of confusing the target analyte with artifacts or extraneous compounds. A major advantage for LAL quantification in the acid hydrolysates is that samples do not have to be derivatized prior to analysis. Unfortunately, ionisation of free amino acids in positive or negative ESI/MS results in the formation of weak molecular ion signals. FMOC and other derivatives have molecular masses that spread far from the interfering protein amino acids, ionise with a lower thermal degradation and give more intense molecular signals in the positive-ion spectra. In samples containing trace amounts of LAL, we did not observe background signals contributing to the peak area of FMOC–LAL (or -*N*-ε-Me-Lys) (not showed). This demonstrates the superiority of the LC–ESI/MS/SIM approach over the LC procedure for detection of LAL. Moreover, casein powders with LAL levels greater than 100 ppm were found to contain no other derivatives than the three FMOC–LAL derivatives, indicating the presence of a constant excess of reagent in the reaction mixture. Therefore, TIC exploration of the LAL–FMOC parent ion or derived fragments should eliminate interference from other compounds or from incomplete reactions. Our outlined procedure did not require any sample manipulation either prior to or after derivatization, only that diagnostic ions of FMOC–LAL are to be measured by ESI/MS–SIM at the correct retention time. Therefore, monitoring of LAL and internal standard derivative ions enhances LAL specificity without the need for preliminary cleanup or prefractionation steps. The best internal standard is deuterated LAL or other stable isotopomer for evaluation of LAL. However, such compounds are currently commercially unavailable, and other compounds were tested as internal standards including the low cost *N*-ε-Me-Lys normally absent from dairy products. In contrast to co-eluting isotopomers, the FMOC-*N*-ε-Me-Lys and -LAL derivatives ionised separately, as they entered the mass spectrometer source at different times. Therefore, relative ion abundance could be accordingly affected by pressure fluctuations, which necessitates the need for carefully controlled and stable mass spectrometer performance. The presence of an internal standard, however, improves assay precision by increasing the limit of quantification irrespective of the sample volume injected. Selected ion monitoring of FMOC–LAL and peak integration formed a unique corresponding molecular mass signal free of interfering masses. The response could be further increased by monitoring two or more fragment ions, in correspondence of the FMOC–LAL derivative peak. In this manner, detection specificity is improved, while sensitivity is lowered. Once integrated, the area of the peak formed by a homogeneous molecular mass signal can easily be compared to that of an internal standard measured under similar conditions. Previously assumed to be a unique and classic criterion, retention time is thus concurrently supplemented with molecular mass determination for ascertaining LAL in dairy products. Unique signals forming each homogeneous peak contained a single defined LAL species, which could be automatically integrated with the appropriate software. Therefore, this outlined procedure is suggested for confirmation of cheese samples once LAL has been found to exceed fixed levels by existing analytical methods.

Acknowledgment

This work was supported by the project GEFORPASTA funded by MIUR-CNR (Italy) – Impiego del Fondo Speciale per lo Sviluppo della Ricerca di Interesse Strategico (Art. 51, comma 9, Legge 27-12-1997, n. 449). The contributing authors have declared no conflicts of interest.

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