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Analysis of trace amounts of bovine β -lactoglobulin in infant formulas by capillary electrophoresis with on-capillary derivatization and laser-induced fluorescence detection

Bovine β -lactoglobulin (β LG) has been described by several authors as the main allergen present in cow's milk. It can induce allergic reactions even at the low concentration existing in hypoallergenic formulas based on hydrolyzed cow's milk proteins (generally lower than μ M). In this paper, the usefulness of a capillary electrophoresis method with on-capillary derivatization and laser-induced fluorescence detection for the analysis of trace amounts of β LG in a commercial hypoallergenic formula has been demonstrated. To confirm the identity of the peak of β LG based on migration time, an immunorecognition step employing an anti- β LG antibody was performed. β LG was quantitated in the whey and casein fractions of the hypoallergenic formula. The concentration of β LG in the whey fraction of the formula was about 3 orders of magnitude lower than the average value present in cow's milk. In the casein fraction of the formula, the concentration of β LG was about 1 order of magnitude lower than in the whey fraction. The method developed was also used for the quality control of three cereal-based infant foods formulated without milk to test the presence or absence of β LG as an indicator of milk contamination during the fabrication process. β LG in a concentration of 10^{-7} M or higher was not observed in any of the cereal-based infant formulas analyzed.

Key Words: On-capillary derivatization; Capillary electrophoresis; Laser-induced fluorescence detection; β -Lactoglobulin; Hypoallergenic formula; Infant formulas; Allergen; LIF; Quantitation

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1 Introduction

Food allergies are estimated to occur in 5 to 20% of children. Allergy to cow's milk proteins is the most common food allergy in early childhood, occurring in 2 to 3% of infants within the first year of life [1, 2].

Clinical manifestations of cow's milk protein allergy include gastrointestinal symptoms, urticaria, angioedema, atopic dermatitis, allergic rhinitis, asthma, or chronic cough [1]. Besides, allergy to cow's milk proteins can be a precursor to other, and sometimes, more serious diseases, such as other food allergies, respiratory allergies or, in the worst cases, even death [1, 3].

Currently, the only effective treatment for cow's milk protein allergy is avoidance of the allergen-containing food.

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Abbreviations: eHF, extensively hydrolyzed hypoallergenic formulas; FQ, 3-(2-furoyl)quinoline-2-carboxaldehyde; β LG, bovine β -lactoglobulin; pHF, partially hydrolyzed hypoallergenic formulas.

In this way, the Food Labelling Directive (Directive 2003/89/EC 25.11.2003) from the European Commission establishes that all ingredients of food products have to be labeled, independently of their proportion in the final product. This directive also contains a list of ingredients liable to cause allergies. However, even if the products are adequately labeled, they can be involuntarily contaminated with allergens. The contamination can occur during the production process, for instance due to a failure in the cleaning system, or during packing or from contamination of ingredients [4]. In addition, these unintentionally added allergens are generally present at low concentrations making their detection more difficult.

During infancy and early childhood a milk substitute is necessary for those children affected by cow's milk protein allergy. Hypoallergenic formulas, based on hydrolyzed cow's milk proteins, have been demonstrated to be the best option for children with cow's milk protein allergy [5]. However, despite the use of hydrolyzed formulas for child nutrition, cases of allergic reactions have been reported for extensively hydrolyzed formulas (eHF) and more commonly for partially hydrolyzed formulas (pHF) [6, 7]. This indicates that minute amounts of residual intact proteins

and/or peptides with a molecular weight high enough to be allergenic are still present in these formulas. More extensively hydrolyzed formulas result in lower residual antigenicity and a lower risk of allergic reactions. However, these formulas suffer from a bitter taste and, sometimes, the high concentration of amino acids present in these products can cause diarrhea in infants. Also, products based on amino acid mixtures have been introduced to avoid cow's milk protein allergy [8]. These products are considered less allergenic than eHF, but the above-mentioned disadvantages are more prominent than in eHF.

Bovine β -lactoglobulin (β LG) is the main allergen present in cow's milk [1, 9]; even at concentrations as low as those present in hypoallergenic formulas (generally lower than μ M) it can generate allergic reactions. The allergenicity of β LG is attributed to several amino acid sequences with a particular three-dimensional orientation within the protein structure [9]. The characterization of these sequences depends on the protein fragmentation method used or the type of receptor studied. Some authors have emphasized the allergenicity of the sequences 41–60, 102–124, and 149–162 [10] or 48–55 [9].

To determine the β LG content in hypoallergenic formulas it is necessary to use high sensitivity methods. HPLC or CE methods with UV detection, although they are fast and high resolution methods, are generally not sensitive enough to determine concentrations lower than μ M. An alternative to UV detection is the use of laser-induced fluorescence (LIF) detection, which provides limits of detection at the nM level or even lower.

In a previous study, a capillary electrophoresis (CE) method with on-capillary derivatization and laser-induced fluorescence (LIF) detection for the analysis of the major whey proteins, bovine serum albumin, α -lactalbumin, and β LG was developed (results published in this special issue of JSS, see page 935). Limits of detection for these proteins were in the range 5–10 nM, 2 orders of magnitude better than those obtained by other authors using CE-UV methods [11, 12].

In this work, the method developed previously was applied to the determination of low concentrations of β LG in a hypoallergenic formula. The β LG content was quantitated in the whey and in the casein fractions of this formula. In addition, the method was applied to the quality control of different types of cereal-based infant foods formulated without milk, in order either to confirm and quantitate or to exclude the presence of β LG as an indicator of milk contamination during the fabrication process. Also, an immunorecognition step for β LG was performed in order to confirm the correctness of the peak assignment for β LG based on the migration time.

2 Experimental

2.1 Reagents, standards, and solutions

Borax, potassium cyanide (KCN), potassium chloride, and β -mercaptoethanol were purchased from Sigma Chemical (St. Louis, MO). Disodium hydrogenphosphate dehydrate, sodium hydroxide (NaOH), sodium chloride, and sodium dihydrogenphosphate were from Merck (Darmstadt, Germany). Hydrochloric acid (HCl) was from Panreac (Barcelona, Spain). 3-(2-Furoyl)quinoline-2-carboxaldehyde (FQ) was from Molecular Probes (Eugene, OR). Methanol was HPLC grade from Scharlau Chemie (Barcelona, Spain). β LG A+B was from Sigma Chemical. Affinity-purified anti-bovine β LG (A+B) raised in rabbit was purchased from Bethyl labs (Montgomery, TX).

Running buffer was 6 mM borax, 6 mM SDS (pH 9.0). A 200 mM KCN stock solution was made in 2.5 mM borax. A 10^{-4} M β LG stock solution was prepared in Milli-Q water (Millipore, Molsheim, France) and stored at 4°C. To carry out the reaction with FQ, a KCN plus β LG solution was prepared by mixing the corresponding volumes of the stock solutions and diluting this mixture with 2.5 mM borax to obtain a final concentration of 10 mM KCN in the mixture. The final concentration of β LG in the mixture depended on the experiment.

Since FQ in solution degraded slowly, even when stored at -20°C in darkness, small aliquots of dried FQ were prepared. To do so, a 50 mM stock solution of FQ was prepared in methanol and 10 μ L aliquots thereof were transferred to 500 μ L microcentrifuge tubes. The solvent was removed under vacuum at room temperature using a model RC10-10 centrifugal evaporator (Jouan, Saint-Herblain, France). The dried FQ was stored at -20°C . It was thawed and dissolved on the day of the experiment.

Safety precautions: Potassium cyanide is highly poisonous and reacts readily with acids to form lethal HCN gas. Stock solutions should be made in a basic buffer. Neutralization of waste containing KCN should be made by addition of 1% NaOH solution (about 50 μ L/g of cyanide) followed by slow addition of bleach (about 70 μ L/g of cyanide).

2.2 Samples

Four commercial samples consisting of a hypoallergenic formula based on partially hydrolyzed whey proteins and three types of cereal-based infant formulas, called type I, II, and III, that were formulated without milk, were analyzed. Main components in these cereal-based infant formulas were: hydrolyzed flour of cereals without gluten (rice, corn, and tapioca) in type I, hydrolyzed flour of cereals (wheat, oats, and corn) and rice protein in type II, and maltodextrin, starch, and several amino acids in type III. In the case of the cereal-based infant formula type I, a

sample collected at the beginning (type I-A) and another one at the end (type I-B) of the manufacturing process were provided by the manufacturer. Besides, the milk (sample C) used by the manufacturer to prepare infant formulas containing milk, and a cereal infant formula containing 32% of milk (sample D) were also analyzed. A type I infant formula guaranteed not to contain milk (sample E) was used as negative control.

2.3 Sample preparation

Samples of the hypoallergenic formula were prepared as follows: 3 g of formula powder was suspended in 10 mL of Milli-Q water. This suspension was heated at 40°C for 30 min using a thermostatic bath (Haake, Karlsruhe, Germany). Six aliquots of 1 mL of the heated solution were centrifuged at $2000 \times g$ for 30 min at room temperature in a Biofuge Stratos centrifuge (Heraeus, Hanau, Germany), cooled in an ice bath for 15 min, and manually skimmed. A 2 M solution of HCl was added until reaching pH 4.6 in order to precipitate the fraction that is non-soluble at this pH, the so-called casein fraction. After that, samples were left to stand for 20 min and then centrifuged at $4500 \times g$ for 15 min at 4°C. The supernatant (whey fraction) and the precipitate (casein fraction) were treated in different ways. The supernatant was collected and filtered through a Whatman 40 filter paper (Maidstone, UK) and through a Tuffryn membrane Acrodisc syringe filter of 0.22 μ m pore size (Pall Corporation, Ann Arbor, MI), successively. This solution was mixed with KCN (in 2.5 mM borax) to a final dilution of 5-fold. The casein fraction was suspended in 0.5 mL of 2.5 mM borax at pH 8.3 plus 15 μ L of β -mercaptoethanol and left to stand for 2 hours at room temperature. The solution was centrifuged at $3000 \times g$ for 15 min and the supernatant filtered through an Acrodisc syringe filter (0.22 μ m). This solution was mixed with KCN (in 2.5 mM borax) to a final dilution of 2-fold.

Samples from cereal-based infant formulas were prepared as follows: 3 g of the infant formula was suspended in 50 mL of Milli-Q water, stirred for 15 min, and then filtered through a Whatman 40 filter paper. A 2 M solution of HCl was added to 6 mL of the filtrate to reach pH 4.6. After that, samples were left to stand for 20 min and then centrifuged at $4500 \times g$ for 15 min at 4°C. The supernatant was collected and successively filtered through a Millex-HV syringe filter of 0.45 μ m pore size with PVDF membrane (Millipore, Bedford, MA) and an Acrodisc syringe filter (0.22 μ m pore size, Tuffryn membrane). This filtrate was mixed with KCN (in 2.5 mM borax) to a final dilution of 2-fold.

Samples of milk (sample C) and samples of the cereal-based infant formula containing milk (sample D) were treated in the same way as cereal-based infant formulas, except that they were skimmed, prior to addition of the

2 M HCl solution, as described for the hypoallergenic formula.

2.4 Instrumentation

The laboratory-made CE apparatus with LIF detection employed was described previously [13]. Briefly, high voltage was provided by a RS/EH50R Glassman power supply. A Spectra Physics Ar-ion laser was used for excitation at 488 nm. Fluorescence was collected at right angles to the laser beam with a 40 $\times$ microscope, filtered successively through a 550 nm cut-off filter and an interference filter centered at 590 nm, imaged onto an iris to block straight light, and detected with a R928 Hamamatsu photomultiplier tube operated at 600 V.

Uncoated capillaries (Composite Metal Services, Worcester, UK) 60 cm length (50 cm to detector) and 50 μ m ID (375 μ m OD) were used. Rinsing of new capillaries and rinsing between runs were performed as described previously [13]. Samples and reagents were introduced into the capillary by gravity (20 cm height) during 12 s. Also, the laboratory-made special device employed to heat 10 cm of the capillary close to the inlet end was described in the above-mentioned work.

2.5 Procedures

FQ was used as the labeling reagent. This compound generates stable fluorescent isoindol derivatives when it reacts with primary amines in the presence of nucleophilic agents, such as KCN [14–16]. The procedure used for on-capillary derivatization was optimized previously (results submitted to this special issue of JSS, see p. 935). Briefly, a plug of a mixture of the sample with a KCN solution at final concentration 10 mM was first injected into the capillary. Next, a plug of a 5 mM solution of FQ in separation buffer was injected. After each injection of sample with KCN or of FQ, the inlet end of the capillary was washed by immersion in a vial containing Milli-Q water. In the next step, vials containing the separation buffer were placed both at the inlet and at the outlet ends of the capillary and a voltage of 3 kV was applied for 6 min. Then, the power supply was switched off for 0.25 min. Reagent mixing and reaction were carried out at 65°C. After reaction, separation was performed at 15 kV at room temperature (the temperature of the room was controlled at 24°C). Each experiment was performed at least in duplicate.

To carry out the immunorecognition step for β LG, 100 μ L of sample was incubated with 1 μ L of anti- β LG antibody (1 mg/mL) in 100 μ L of a phosphate-buffered saline solution (PBS = 0.01 M sodium phosphate, 0.138 M sodium chloride, 0.0027 M potassium chloride, pH 7.4) in a horizontal shaker (Ika Labortechnik, Staufen, Germany) at 250 rpm for 1 h at room temperature. After that, samples

were passed through a Microcon cartridge (Amicon, Bedford, MA) of 50000 Da cut-off membrane by centrifugation at $12000 \times g$ for 15 min at room temperature. The filtrate was recovered and fluorescently derivatized on the capillary. A control assay to which the antibody had not been added was performed in parallel.

3 Results and discussion

It has been recorded that heating of milk (even under pasteurization conditions) can result in a loss of β LG in the whey fraction [17]. In such cases, β LG can form a complex with caseins via disulphide bridges and, therefore, it would be present in the casein fraction. As a consequence, in order to know the possible allergenicity of the hypoallergenic formula, due to the presence of β LG, it is necessary to determine the allergen concentration in both the whey and the casein fractions. The electropherograms obtained from the whey and casein fractions of the hypoallergenic formula using the method developed are shown in **Figure 1**. The relative standard deviation for the migration time of β LG was around 1%. To identify the peak corresponding to β LG, samples were spiked with a standard solution of β LG at final concentration 3×10^{-7} M.

To confirm that the peak assignment to β LG corresponds to this protein, an immunorecognition step was carried out for β LG. The aim of this assay was to form a [β LG + anti- β LG] complex that can be separated from free β LG on the basis of their different molecular weights (18 kDa β LG, 150 kDa anti- β LG, and >150 kDa [β LG + anti- β LG] complex). A cartridge with a cut-off membrane of 50 kDa, that allowed the retention of high molecular weight molecules, was used for this purpose. Therefore, if β LG in the sample is able to form a complex with anti- β LG, this complex would be retained in the 50 kDa cut-off membrane and β LG should not be detected in the fraction filtered through the membrane. A control assay, in which the antibody was not added, was performed in parallel in order to exclude the possibility that β LG would not be detected in the filtration fraction due to losses of β LG during sample manipulation in the cartridge, for instance by adsorption.

To carry out this experiment, it is necessary that free β LG passes through the 50 kDa cut-off membrane and the antibody does not generate peaks in the filtrate that could interfere with the β LG peak. To prove that free β LG passes through the cartridge, a solution 2.5×10^{-7} M of β LG in PBS buffer was passed through the cartridge under the above-mentioned conditions and the filtrate fraction was recovered. Besides, the retentate fraction was recovered by inverting the cartridge and centrifuging it at $1000 \times g$ for 3 min. To increase the recovery, this last step was repeated after adding 10 μ L of water to the membrane. Both fractions, filtrate and retentate, were injected onto the capillary. A peak for β LG was obtained in the

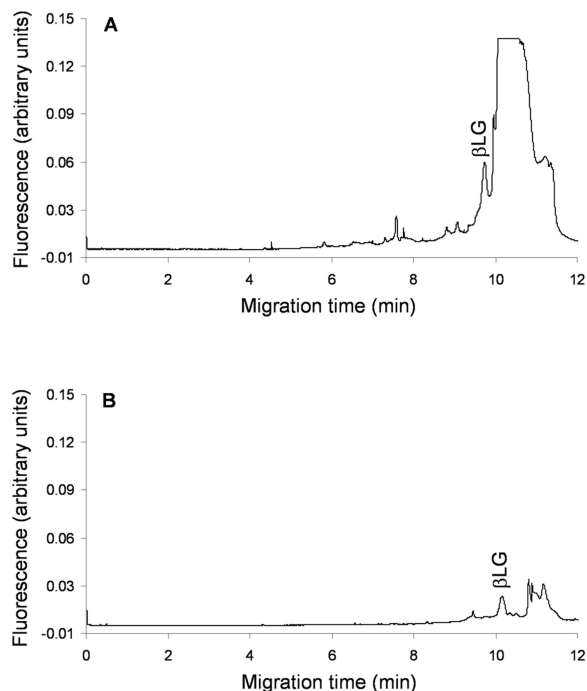


Figure 1. Separation of β LG in a sample of the hypoallergenic formula: (A) whey and (B) casein fraction. Conditions: running buffer 6 mM borax-6 mM SDS at pH 9.0, uncoated capillary ($L = 60$ cm, $l = 50$ cm, 50μ m ID), injection by gravity ($h = 20$ cm, 12 s) of a mixture of the sample with a 10 mM KCN solution followed by an injection of 5 mM FQ in running buffer, mixing time 6 min at 3 kV, reaction time 0.25 min at 0 kV, mixing and reaction temperature 65°C , separation performed at 15 kV at 24°C .

electropherogram from the filtrate fraction. In the case of the retentate fraction, no peak was observed for β LG. Therefore, no free β LG is retained on this cartridge. A control experiment, in which one aliquot of the same β LG solution was not passed through the cartridge, was performed in parallel to detect a possible loss of β LG by adsorption in the cartridge. The peak height obtained for β LG was slightly higher (2.6%) in the control sample than in the filtrate fraction, indicating that a small amount of β LG could be adsorbed in the cartridge (results not shown). On the other hand, a 0.01 mg/mL solution of anti- β LG antibody in PBS buffer was passed through the cartridge and the filtrate fraction recovered and injected onto the capillary. Besides, a control experiment in which the antibody was not passed through the cartridge was carried out in parallel. In both cases, the electrophoretic profile obtained was similar to that obtained in a blank assay (in which anti- β LG was not added to the PBS solution), and it was therefore concluded that the presence of the antibody in the sample did not generate possible interference peaks (results not shown).

After these preliminary studies, incubation of the whey of the hypoallergenic formula and the antibody was carried

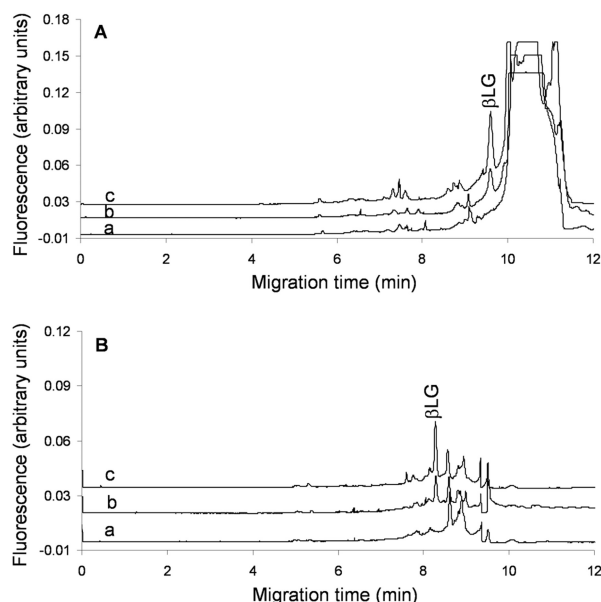


Figure 2. CE profiles obtained from the hypoallergenic formula: (A) whey fraction and (B) casein fraction under the following conditions: (a) incubated with anti- β LG, (b) non-incubated with anti- β LG, and (c) non-incubated with anti- β LG and spiked with standard β LG. Other conditions as in Figure 1.

out, the mixture filtered through a cartridge of 50 kDa cut-off membrane, and the filtrate injected into the capillary. As expected, no peak was observed at the migration time corresponding to β LG, due to the formation of the immuno-complex and its retention on the membrane of the cartridge. This result indicates the correctness of the assignment of the peak for β LG made previously (see **Figure 2.A**).

A similar experiment was carried out for the casein fraction of the hypoallergenic formula. The casein fraction, previously solubilized using β -mercaptoethanol, was incubated with the antibody under the same conditions as the whey fraction. As occurred with the whey, at the migration time corresponding to β LG, no peak was observed in the electropherogram corresponding to the casein fraction incubated with the antibody, while in the electropherogram corresponding to the control sample (without antibody) a peak for β LG was observed (see **Figure 2.B**). These experiments were carried out using a new capillary, which accounts for the differences in migration time of β LG observed between Figure 2.B and previous figures.

The developed method was applied to the quantitation of β LG in the whey and casein fractions of the hypoallergenic formula. Quantitation of β LG in the casein fraction is also necessary since this protein can be present in this fraction due to the processes carried out during the manufacturing of the hypoallergenic formula. Using peak height, a linear

calibration curve was obtained in the range 10^{-6} – 10^{-7} M (correlation coefficient of 0.9992). The β LG content found in the whey of the formula was 3.16×10^{-9} mol/g (4.46×10^{-7} M), about three orders of magnitude lower than the average value of β LG in whey of non-processed milk, 1.8×10^{-4} M [18]. Our result is in agreement with a result obtained in our laboratory for the analysis of β LG plus its peptides larger than 10 kDa in the whey of the same hypoallergenic formula using an immunochromatographic method, that was 2.75×10^{-9} mol/g (unpublished results). The β LG content that we found in the casein fraction of the formula was 3.42×10^{-10} mol/g (4.83×10^{-8} M), about one order of magnitude lower than the amount of β LG in the whey fraction. The method developed was useful to quantitate the β LG content in both the whey and the casein fractions.

In another experiment, three types of cereal-based infant foods formulated without milk were analyzed for quality control purposes. The aim of this assay is to determine whether β LG is present, as an indicator of milk contamination during the fabrication process when the same manufacturing facilities were used for other products containing milk. The same samples were spiked with a standard solution of β LG at a final concentration of 10^{-7} M. For spiking with milk, 10% (w/w) of milk was added to these samples before sample treatment. In all cases, the fraction soluble at pH 4.6 (equivalent to the whey in milk) was analyzed.

If samples I, II, or III were contaminated during the fabrication process, contamination should come from cereal infant formulas containing milk previously manufactured in the same facilities. In order to know whether the electrophoretic behavior of β LG is modified by the fabrication process or by the matrix, the electrophoretic profiles of the whey fraction of milk (sample C) and of the cereal infant food formulated with milk (sample D) were obtained (results not shown). Identification of β LG was carried out by spiking with the standard protein. The presence of β LG in both kinds of samples was observed. Thus, if samples not intended to contain milk were contaminated, identification of β LG in them would be possible.

To prove the absence of any peaks in the zone of the electropherogram corresponding to β LG in sample E (infant formula type I guaranteed not to contain milk), a comparison was made between the electropherograms obtained from a blank (assay in which sample was substituted by borax 2.5 mM), sample E, sample E spiked with a standard solution of β LG, and sample E spiked with milk (sample C) (see **Figure 3**). As expected, there was no peak corresponding to β LG in sample E. A peak for β LG was observed in sample E spiked with standard β LG or with milk.

The samples of cereal-based infant formulas type I-A, type I-B, and type II were then analyzed. For each sam-

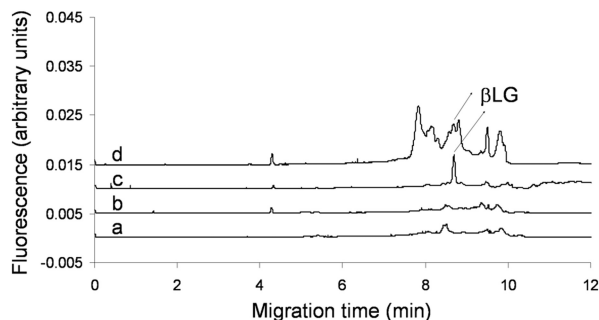


Figure 3. CE profiles obtained from (a) a blank assay, (b) an infant formula type I guaranteed not to contain milk, (c) same sample as in (b) but spiked with standard β LG, and (d) same sample as in (b) but spiked with milk. Other conditions as in Figure 1.

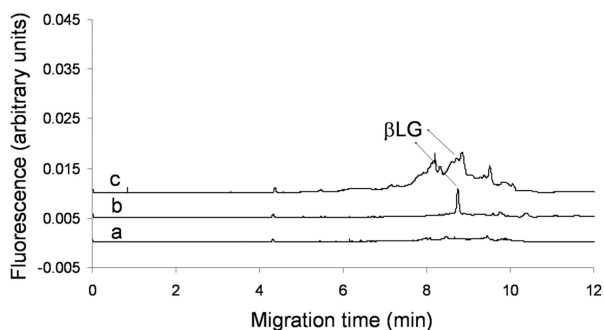


Figure 4. CE profiles obtained from (a) a sample of an infant formula type II, (b) same sample as in (a) but spiked with standard β LG, and (c) same sample as in (a) but spiked with milk. Other conditions as in Figure 1.

ple, a comparison was made between the electropherograms from the sample, the sample spiked with a standard solution of β LG, and the sample spiked with milk. As an example, **Figure 4** compares electropherograms for the infant formula type II. β LG in concentrations of 10^{-7} M or higher was not found in any of the infant formulas studied.

Electropherograms corresponding to the analysis of infant formula type III and the same sample spiked with standard β LG and with milk were obtained (see **Figure 5.A**). In contrast to the case of samples types I and II, several peaks appear in the electropherograms in the zone of the β LG peak. This precludes identification of β LG in this infant formula on the basis of migration time. To eliminate the maximum amount of interfering peaks due to small-size compounds, a Microcon (Amicon) cartridge of 3000 Da cut-off membrane was employed. Samples were centrifuged in this device at $13000 \times g$ for 20 min and the retentate (fraction >3 kDa) was recovered by inverting the cartridge and centrifuging it at $1000 \times g$ for 3 min. To increase the recovery, this step was repeated after adding $10 \mu\text{L}$ of water to the membrane. The number of peaks appearing in the electropherogram of the fraction >3 kDa

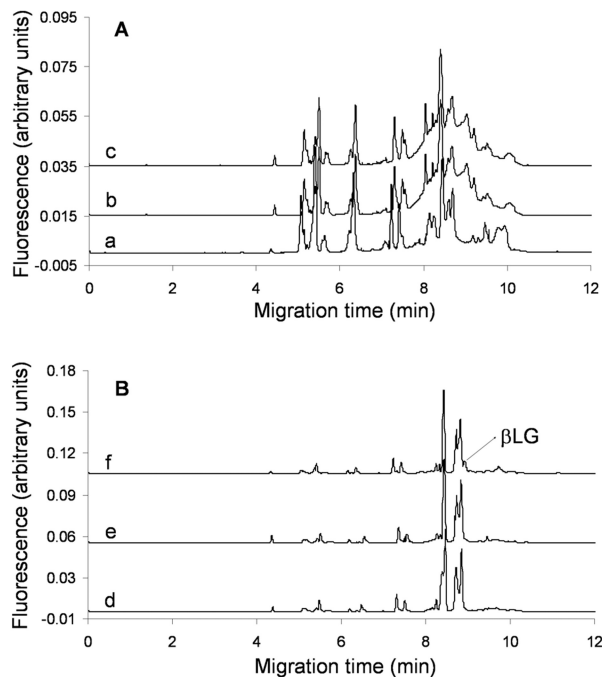


Figure 5. CE profiles obtained from (A) samples of the infant formula type III: (a) non-spiked, (b) spiked with standard β LG, (c) spiked with milk, and (B) filtrates of samples of the infant formula type III in PBS through a 50 kDa membrane: (d) incubated with anti- β LG prior to the filtration step, (e) non-incubated with anti- β LG, and (f) non-incubated with anti- β LG and spiked with standard β LG after the filtration step. Other conditions as in Figure 1.

is similar to that appearing in the total sample (results not shown). Probably, most of these peaks correspond to compounds with high molecular weight that cannot be separated from β LG in this cartridge. As the cartridge used did not allow us to resolve the difficulties associated to the identification of β LG in sample type III, an immunorecognition step was carried out. To do this, sample type III was incubated with anti- β LG following the method described for the hypoallergenic formula. The electropherogram obtained was compared with that obtained in a control sample in which the antibody was not added. No differences were found between these electropherograms. This indicates that β LG was not present in the sample and none of the peaks corresponds to this protein. This result was confirmed by spiking the control sample, with a standard solution of β LG (see **Figure 5.B**). It is clearly seen that the peak corresponding to β LG does not correspond to any of the peaks present in the non-spiked sample.

4 Concluding remarks

The suitability of a capillary electrophoresis method with on-capillary derivatization and laser-induced fluorescence

detection for the analysis of trace amounts of β LG in a commercial hypoallergenic formula has been demonstrated. The initial assignment of the peak of β LG based on migration time was confirmed by an immunorecognition step employing anti- β LG. β LG was quantitated in both the whey and the casein fractions of the hypoallergenic formula. The total β LG content can be useful to estimate the potential allergenicity of this hypoallergenic formula and other foods.

The method developed was also suitable for confirming the absence of β LG as an indicator of milk contamination during the manufacturing process in three cereal-based infant foods formulated without milk. This method could probably be successfully applied to the detection of β LG as an indicator of milk contamination in other types of food.

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