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A new sample preparation method compatible with capillary electrophoresis and laser induced fluorescence for improving detection of low levels of β-lactoglobulin in infant foods.

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Abstract

Beta-lactoglobulin (βLG) is the main allergenic protein in cow's milk and can cause allergy even when present at very low concentration. The aim of this work is to develop an innovative sample preparation method fully compatible with capillary electrophoresis and laser induced fluorescence detection for improving the sensitivity when analyzing βLG. Different types of baby food were on purpose contaminated with diverse dairy desserts and submitted to thermal treatment to simulate potential contamination at production. Sample preparation prior to CE analysis was performed by the classical extraction method and by the innovative one, and the results were compared. Analysis was performed by capillary electrophoresis with laser-induced fluorescence detection. The innovative method permitted to detect contaminations as low as 1 part of yoghurt in 10 000 parts of baby food.

Keywords: Capillary electrophoresis; laser-induced fluorescence detection; extraction method; β-lactoglobulin; allergen; baby food.

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1. Introduction

Food allergy is now recognized as a worldwide problem and it seems to be on increase. In the US food allergy affects up to 5% of children less than 5 years of age and approximately 4% of the general population [1].

The immunologic reaction to milk proteins is considered to be the most common food allergy. Some milk allergic individuals, especially infants, are sensitive even to traces of cow's milk proteins in commercial foods that should not contain dairy products. Some allergic individuals may not experience a reaction until the ingestion of more than 10 grams of milk while others are sensitive to less than one milligram [2]. Up to now, an avoidance diet is the only preventive measure available to sensitized individuals [3,4]. Milk allergy is also of concern in other fields not related to food production. For instance, milk proteins are frequently used as ingredients in the cosmetics and pharmaceutical industries and can cause allergic responses in milk-sensitive patients [5,6]. In addition, the production of bio-pharmaceutical products in the milk of transgenic cows demands the determination of milk proteins due to its allergenicity [7]. For this reason growing interest has been shown in detecting masked allergens which are present in such small doses as to be almost undetectable.

In some cases the presence of these allergens in foodstuff is the result of involuntary contamination related to the production process [8]. Food companies, using the same production line for manufacturing food containing and non-containing milk, have to be sure that there is not cross-contamination with dairy products along the manufacturing process.

The knowledge of milk presence in baby food is important for a more qualified nutritional recommendation to parents with children susceptible to allergies and for quality assurance of baby food claimed to be free of milk. For these reasons an accurate labeling of commercial products is desirable. According to the latest EU Labeling Directive (Directive 2007/68/EC 27.11.2007), milk and its derivatives must be labeled in commercial foods [9].

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Bovine β-lactoglobulin (βLG) has been described as the main allergenic protein in cow's milk [10] even when present at low concentrations. Level of detection of βLG in a sample depends on the analytical method used. Several methods for the determination of βLG in food products have been published. Presently, the ELISA technique is used in routine food analysis [8]. Although classic ELISA is sensitive, selective, and it allows the analysis of several samples simultaneously, this method is tedious and time consuming, and sometimes provides only semi-quantitative data. Immunoassays performed in HPLC format have been applied to determine residual βLG in hypoallergenic infant formulas [11]. HPLC or CE methods with UV detection, although being fast and high resolution methods, are not sensitive enough to determine concentrations of proteins at the nM level. An alternative to UV monitoring is the use of laser-induced fluorescence (LIF) detection, which provides for proteins, fluorescently derivatized and analyzed for CE-LIF, limits of detection at the 10^{-9} M level or even lowers [12]. Several techniques for protein derivatization for LIF detection using fluorescent or fluorogenic reagents performed by off- or on-column methods can be used [13].

In a previous study carried out in our laboratory, CE-LIF was used to analyze trace amounts of βLG in a commercial hypoallergenic formula and for the quality control of cereal-based infant formulas [14]. In that study sample preparation for βLG analysis was performed following classical methods [15]. A study recently carried out in our laboratory demonstrated, using ELISA, that the procedure followed for sample preparation prior to the analysis has a noticeable effect on the sensitivity of the assay [16].

The objective of this work has been to modify the sample preparation method developed in the previous study [16] to try to increase, even more, the sensitivity and the speed and to study the compatibility of the optimized sample preparation method with the analysis of βLG by CE-LIF. Samples of baby food of three types, based on fruit, fish, or poultry and on-purpose contaminated with dairy desserts containing three different dairy products were prepared and submitted to thermal

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treatment to simulate the cross-contamination that potentially could happen during in the manufacturing line. Those samples were submitted in parallel to the classical and the new sample preparation methods, the extracts were analyzed by CE-LIF, and the results of both sample preparation methods were compared.

2. Experimental

2.1. Reagents and standards

Sodium tetraborate decahydrate (borax), guanidine hydrochloride, potassium chloride, potassium cyanide, o-phenylenediamine (OPD), rabbit serum, and β-mercaptoethanol were purchased from Sigma (St. Louis, MO, USA). Disodium hydrogenphosphate, sodium chloride, sodium dihydrogenphosphate, sodium hydroxide, sodium dodecyl sulphate (SDS), and Tween 20® were from Merck (Darmstadt, Germany). Sulphuric acid and sodium carbonate were from Panreac (Barcelona, Spain). Concentrated buffer with stabilized hydrogen peroxide for enzymatic reaction with horseradish peroxidase was purchased from Pierce (Rockford, IL, USA). 3-(2-Furoyl)quinoline-2-carboxaldehyde (FQ) was from Molecular Probes (Eugene, OR, USA). β-Lactoglobulin A+B (βLG) was from Sigma. Affinity purified anti-bovine βLG (A+B) raised in rabbit (anti-βLG) unconjugated and conjugated with horseradish peroxidase (anti-βLG-HRP) was purchased from Bethyl labs (Montgomery, TX, USA). Methanol was HPLC grade from Scharlau Chemie (Barcelona, Spain). Water from a Milli-Q water system (Millipore, Bedford, MA, USA) was used.

A 200 mM KCN stock solution was made in 2.5 mM borax. To carry out the reaction with FQ, a solution containing KCN and βLG (standard βLG or βLG extracted from the samples) was

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prepared by mixing the corresponding volumes of the KCN stock solution and βLG and diluting this mixture with 2.5 mM borax to obtain a final concentration of 10 mM KCN in the mixture.

A 50 mM stock solution of FQ was prepared in methanol. Since FQ in solution degrades slowly, even when stored at -20ºC in darkness, small aliquots of dried FQ were prepared. To do so 10 μL aliquots of the methanolic solution were transferred to 500 μL 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 ºC until use.

A 10^{-4} M stock solution of Rhodamine B was prepared in water. This solution was kept away from light to avoid degradation.

OPD solution (0.5 mg mL^{-1}), substrate for HRP, was prepared everyday in buffer containing stabilized H_2O_2. This buffer was prepared in the moment of use from the 10× concentrated commercial solution.

Standard aqueous solutions of βLG in the range 5×10^{-10} - 1×10^{-7} M were prepared from a 1 mg mL^{-1} stock solution. The aqueous stock solution was aliquoted in low binding polymer vials (Sorenson BioScience, Salt Lake City, UT, USA) and stored at - 4 ºC.

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 followed by addition of bleach.

2.2. Samples

Baby foods, named from now on as FRUIT, FISH, and MEAT types, were analyzed. Main components in these matrices were: orange and banana with cereal in type FRUIT, hake with rice in type FISH, and chicken with rice in type MEAT. Samples named as FRUIT0, FISH0, and MEAT0 corresponded to baby foods guaranteed free of dairy products. These matrices were contaminated

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on-purpose with known amounts of previously processed dairy desserts to simulate the contamination that could happen during the manufacturing processes. These dairy desserts were yoghurt with pear, fresh cheese with fruit, and rice pudding. After on-purpose contamination, the baby foods were submitted to the same thermal treatment that they would experience if contamination had happened in real production process. That is, 10 min at 105 ºC for FRUIT samples, 45 min at 123 ºC for FISH samples, and 50 min at 121 ºC for MEAT samples.

The yoghurt with pear dessert employed as contaminant contained 26% of yoghurt. The fresh cheese with fruit dessert contained 40% of fresh cheese. The rice pudding contained 65% of milk.

Proportions of dairy desserts and their corresponding content in dairy products added to each of the samples studied are indicated at Table 1.

2.3. Sample preparation

For the isolation of βLG from the samples, the classical method employed for extracting the whey fraction [15] was compared to the new method optimized in this work. Schemes of both sample preparation methods, *method 1* and *method 2*, are shown in Table 2.

The classical method, from now on *method 1,* was as follows: 1 g of the baby food was suspended in 10 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 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 at room temperature and then centrifuged at 4500 × *g* for 20 min at 4 ºC. The supernatant (whey fraction) was collected and successively filtered through a Millex syringe filter PVDF membrane of 0.45 µm pore size (Millipore, Bedford, MA, USA) and through a Tuffryn membrane Acrodisc syringe filter of 0.22 µm pore size (Pall Corporation, Ann Arbor, MI, USA).

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The new method was optimized by studying, using ELISA, the influence of several factors on sensitivity. A method recently developed in our laboratory for βLG extraction was taking as starting point [16]. This previously developed method was as follows: 1 g of the sample was transferred to a 15 mL polypropylene tube and suspended in 10 mL of concentrated buffer saline (PBS×10, this is, 0.1 M phosphate buffer, 1.38 M NaCl, 0.027 M KCl, pH 6.85) and the fat on the surface was removed. An aliquot of 500 μL of the extracting solution containing a final concentration of 24 mM β-mercaptoethanol, 25 mM guanidine hydrochloride and 5% (v/v) of 2.5 mM borate buffer at pH 8.3 was added to the tube containing the sample. The tube was mixed thoroughly and shaken for 2 h at room temperature. The suspension was diluted in 30 mL of 0.15 M sodium chloride and shaken for 1 h at room temperature. The tube was centrifuged for 35 min at 9000 × g at room temperature. The supernatant was successively filtered through a Whatman® 40 filter paper and through a Millex®HV syringe filter PVDF membrane of 0.45 μm pore size and transferred to clean Eppendorf tubes. Analysis by ELISA was performed within 24 h of extraction [16,17].

In the present work the influence of temperature of the extraction step, extraction time, skimming, and simultaneous versus sequential addition of components of the extracting solution skimming, simultaneous versus sequential addition of components of the extracting solution, and temperature and time of the extraction step have been studied to obtain a method with higher sensitivity, speed, and simplicity.

2.4. Instrumentation and devices

Microtiter plates (Immuno MaxiSorp 96-MicroWell plates, NUNC, Rochester, NY, USA) with C bottom shape were used to perform the ELISA. The absorbance in the ELISA plates was measured at 492 nm in a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

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The laboratory-made CE apparatus with LIF detection employed was the one described previously [18] with small modifications. Briefly, high voltage was provided by an RS/EH50R power supply (Glassman High Voltage, Whitehouse Station, NJ, USA) used in the normal polarity configuration (anode connected at the inlet end of the capillary). A 2060-10S Spectra Physics Ar-ion laser (9 mW) (Spectra Physics, Mountain View, CA, USA) was used for excitation at 488 nm. Fluorescence was collected at right angle to the laser beam with a 40x microscope objective, 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 an R928 photomultiplier tube (Hamamatsu, Hamamatsu City, Japan) operated at 600 V and assembled on top of a high-precision stage for alignment. Photocurrent was processed by a 7070 photometer (Oriel, Stratford, CT, USA) and a 406 System Gold A/D converter (Beckman, Fullerton, CA, USA). Data were collected on a 486 computer. A laboratory-made special device was used to heat a small zone (10 cm) at the inlet of the capillary where the derivatization reaction took place, using an F3 thermostatic bath (Haake, Karlsruhe, Germany).

Uncoated capillaries (Composite Metal Services, Worcester, UK) 60 cm length (50 cm to the detector) and 50 μm I.D. (375 μm O.D.) were used. Centrifugal filter devices Microcon YM-50 (Amicon, Beverly, MA, USA) with a cut-off membrane of 50 kDa were used for the immunorecognition procedure.

2.5. ELISA method

Enzyme-linked immunosorbent assays in sandwich format were carried out under the conditions previously optimized [16].

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Separation buffer was 6 mM borax, 6 mM SDS (pH 9.0).

New capillaries were rinsed with 1 M NaOH (100 μL) followed by a rinse with Milli-Q water (100 μL). Between runs, the capillary was sequentially rinsed with Milli-Q water (100 μL), 0.1 M NaOH (100 μL), Milli-Q water (100 μL), and the separation buffer (100 μL). Rinses were made manually employing a model 1710 glass syringe (Hamilton, Bonaduz, Switzerland).

FQ was used as the labeling reagent because it generates stable fluorescent derivatives when reacts with primary amines in the presence of nucleophilic agents, such as KCN [19]. The procedure used for on-capillary derivatization was based on that one optimized previously employing a mixture of three standard proteins [20]. Namely Briefly, a plug of a mixture of the sample plus a KCN solution at final concentration 10 mM, was injected into the capillary. Next, a plug of a 5 mM solution of FQ in separation buffer was injected. Both injections were performed by gravity (20 cm height) during 12 s. After each injection of either sample plus KCN or FQ, the inlet end of the capillary was washed by immersion in a vial containing Milli-Q water. Next, vials containing the separation buffer were placed in the inlet and in the outlet ends of the capillary and a voltage of 3 kV was applied for 6 min (mixing step). Then, the power supply was switched off for 15 s (reaction step). Reagents mixing and reaction were carried out at 65 ºC. After reaction, separation was performed at 15 kV at room temperature (the temperature of the room was controlled to be 24 ºC). Each experiment was made, at least, in duplicate.

The immunorecognition step for βLG was performed as follows [14]: one hundred μL of sample were incubated with 1 μL of anti-βLG antibody (1 mg mL^{-1}) in 100 μL of PBS in a horizontal shaker at 250 rpm for 1 h at room temperature. After that, samples were passed through a Microcon centrifugal filter device of 50 kDa cut-off membrane by centrifugation at 12 000 × *g* for 15 min at room temperature. The filtrate was recovered and derivatized with FQ on the capillary for its CE-LIF analysis. A control assay in which the antibody had not been added was performed in

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parallel. Prior to their use the centrifugal filter devices were passivated overnight with a 5% Brij 35 solution (w/v) to avoid the adsorption of proteins [21].

Spiking with a large concentration of βLG for confirmation of the identity of the CE peak was carried out by adding a known volume of standard 10^{-4} M βLG to the solution extracted from the sample in order to obtain the desirable concentration of standard in the sample plug. The spiked solution was injected and derivatized on the capillary.

3. Results and discussion

3.1. Optimization of the sample preparation method

Firstly, the influence of carrying out extraction at different temperatures higher than room temperature was studied. The temperatures assayed were 37, 50, 70, 80, and 90 ºC. The optimization was carried out with sample MEAT1 and the effect observed was checked afterwards for the temperature selected with samples FISH1 and FRUIT1. The results obtained are shown in Table 2. It was observed (data not shown) that when increasing the temperature at 80 ºC or higher the concentration of βLG detected by ELISA was increased. For temperatures of 70 ºC or lower no differences were observed by respect to room temperature. Between 80 and 90 ºC no differences were observed, so 80 ºC was selected as the temperature of the extraction. The βLG content detected in the samples MEAT1 and FISH1 extracted at 80 ºC was about 5 times and 3 times higher, respectively, to that obtained when extraction was performed at room temperature. For sample FRUIT1 extraction at 80 ºC led to an increase of two orders of magnitude for the βLG concentration detected in comparison to the one found when extraction was carried out at room temperature.

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Afterwards, with the aim of simplifying the sample preparation method the effect of suppressing the skimming step was tested in samples MEAT1, FISH1, and FRUIT1. Decrease in the sensitivity was not observed (results not shown). Thus, the skimming step was eliminated.

In the next step of the optimization, the influence of performing simultaneous instead of sequential addition of all the components of the extracting solution was studied (see Table 2). When addition was carried out sequentially (2 steps process), first, β-mercaptoethanol, guanidine hydrochloride and borate buffer were added to the tube containing the sample and shaken for 2 h at room temperature. In a second step, the suspension was diluted with 30 mL of 0.15 M sodium chloride and shaken for 1 h at room temperature. For simultaneous addition (1 step process) all of the components (β-mercaptoethanol, guanidine hydrochloride, borate buffer and 0.15 M sodium chloride) were added together followed by 3 h of shacking. As shown in Table 2 no No differences in the concentration of β-LG detected were obtained for any of the three types of samples between the sequential and the simultaneous addition modes, thus the simultaneous addition was selected.

Finally, on an effort for reducing the time needed for extraction, the effect of the extraction time in the range 3 hours- to 15 min was studied, observing for three types of samples that a reduction on the time did not have influence on the βLG detected by ELISA (see Table 2). Thus, 15 min was selected for the extraction.

It may be concluded from this optimization that the new protocol, named as *method 2* in this work, permits to increase the sensitivity of the detection of βLG. A comparison between the concentrations detected by ELISA by *method 1* and *method 2* is shown in Table 3. *Method 2* allows also decreasing to about one half the sample preparation time (95 min for *method 1 vs* 50 min for *method 2*).

As a result, the optimized method, *method 2*, was as follows: 1 g of the baby food was suspended in 10 mL of concentrated phosphate buffered saline (PBS 10 ×). Five hundred μL of the extracting solution (final pH 7.3) containing 24 mM β-mercapthoethanol, 25 mM guanidine

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hydrochloride and 5% of 2.5 mM borate buffer at pH 8.3 were added to the suspension followed by 30 mL of 0.15 M sodium chloride. The mixture was shaken for 15 min at 80 ºC and then centrifuged at 9000 × *g* for 25 min at room temperature. The supernatant was collected and successively filtered through a Whatman 40 filter paper and through a syringe filter PVDF membrane 0.45 μm pore size.

3.2. Analysis of the extracted samples by CE-LIF

Characteristics of the CE-LIF method

Firstly, the characteristics of the CE-LIF method described were studied. On-capillary derivatization and CE separation conditions were optimized in a previous paper of our group [14]. The separation buffer contains SDS, which its main role is to minimize band broadening due to the multiply labeled reaction products of FQ with the proteins, mainly through their Lys residues [19]. The repeatability (run-to run precision) for migration time and peak height was calculated as the relative standard deviation (RSD) for 12 injections of standard βLG. The repeatability was 1.21% for migration time and 5.12% for peak height. The detection limit (LOD) for standard βLG calculated for a signal-to-noise ration of 3 was 5×10^{-10} M correspondent to the minimum amount of unlabeled protein that should be present in the sample to be detected. This LOD is one and a half orders of magnitude better than that previously obtained in our laboratory using the same method for this protein [20] and three and a half orders better than those obtained using CE-UV methods [22, 23].

Selectivity of the method

To test the compatibility of the new sample preparation method with the CE-LIF method for the analysis of βLG the influence of the extracting agents employed in *method 2* on the signal obtained by CE-LIF was studied. For this purpose, the electropherograms of the agents employed in

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the extracting solution were individually and jointly compared to the electropherograms of the extracts of the baby food samples of the three types of matrices studied. The electropherograms of β-ME and guanidine hydrochloride, and the mixture of both agents were obtained by using them as sample in the CE-LIF procedure. It could be observed that neither β-ME, nor guanidine hydrochloride, nor their mixture produced any peak in the electropherogram (data not shown). Thus, the use of β-ME and guanidine hydrochloride did not interfere with the detection of βLG in the samples and the sample preparation method developed was in this sense compatible with the CE-LIF method.

Also, the influence of the components of the baby food matrix on the signal of βLG on the electropherograms was tested. To do so, the electropherograms of the extract of the sample of type FRUIT guaranteed milk free of the three different matrices (FRUIT0, FISH0 and MEAT0) were individually was compared to the baby food of the same matrix adulterated with dairy dessert (FRUIT2). The electropherogram of the extract obtained by *method 2* of sample FRUIT2 showed a small peak migrating in front of a very large broad peak. The extract of the sample FRUIT0, guaranteed to be free of milk, did not show any peak in front of the large broad one. Spiking of the extract of FRUIT2 with 5×10^{-7} M standard βLG gave rise to an increase of the height of the small peak, which then was tentatively assigned to βLG. Migration time (tm) of the large peak was different between samples FRUIT2 and FRUIT0 (results not shown). Change in migration time in the sample containing yoghurt versus the one non containing it could be due to different facts, such as, the change in ionic strength or the presence of some compounds.

Identification of βLG peak

After these preliminary results, in order to discard false positives or false negatives due to differences in the migration time, an internal standard (I.S.) was introduced in the sample plug to obtain a more robust migration parameter.

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Rhodamine B (RhB) was assayed as I.S. because of its solubility in aqueous solvents and its maximum of fluorescence emission around 590 nm when excited at 448 nm [24]. The final concentration of RhB injected was 10^{-7} M.

RhB migrated as a single peak under the CE-LIF conditions used. Its addition to the sample as I.S. showed that the peak did not interfere with any component of the baby food sample and it had a migration time close to and shorter than the βLG peak. As it can be observed in Figure 1 and in Table 4 the migration time of the I.S. was different in the sample which contained dairy dessert (FRUIT2) than in the one free of milk (FRUIT0); also as mentioned above, the migration time of the large peak was different in both samples. The migration time of the I.S. and of the large peak were not modified when the extract of FRUIT0 was spiked with standard βLG. For this spiked sample the ratio $tm/tm_{I.S.}$ for βLG was 1.047. The value was very close to that obtained ($tm/tm_{I.S.}$ = 1.045) when standard βLG was analyzed in the absence of the baby food. The average value (n = 20) of the relative migration of the small peak of the extract of sample FRUIT2 that had been tentatively assigned to βLG was $tm/tm_{I.S.}$ = 1.046 (see Table 4). This value for the relative migration time reinforces the assignment of the peak to the allergenic protein. In comparison, as expected, no peak was observed in the electropherogram of FRUIT0 (sample guaranteed free of βLG) at $tm/tm_{I.S.}$ about 1.046, which would had corresponded to βLG (Fig. 1B and Table 4). Thus, the components of the baby food type FRUIT did not comigrate with the βLG peak in the electropherograms and they did not interfere on the detection of βLG. The βLG content in this sample was shown to be below the detection limit (5×10^{-10} M). Besides reinforcing the assignment of the βLG peak, the use of the I.S. markedly improved the repeatability (RSD = 1.02 % for $tm_{\beta LG}$ *vs* RSD = 0.12 % for $tm_{\beta LG}/tm_{I.S.}$).

To definitely confirm the correctness of the assignment of the peak for βLG, an immunorecognition step was carried out. For this purpose, a centrifugal filter device with a 50 kDa cut-off membrane, that allowed the retention of the complex [βLG + anti-βLG] (molecular weight

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of antibody is about 150 kDa) was used. Therefore, if there is βLG in the extract of the sample, the complex would be retained in the membrane and βLG should not be detected in the filtrate fraction. A control assay, in which the antibody was not added, was performed in parallel in order to exclude the possibility that βLG would not be detected in the filtrate due to losses of βLG during manipulation. Both filtrates, the one from the extract of the sample incubated with the antibody and the one from the extract of the sample non-incubated with the antibody, were analyze by CE-LIF. As expected, the peak assigned to βLG was not observed in the electropherogram obtained from the filtrate of the sample incubated with the antibody and it appeared in the electropherogram of the filtrate of the sample non-incubated with the antibody (results not shown). This result confirms the assignment of the peak corresponding to βLG.

Comparison of method 1 vs method 2

Once observed the feasibility of the analysis method to unequivocally identify the peak of βLG in the electropherograms for the extract of the baby food sample FRUIT2 prepared using the innovative extraction method, the efficiency of both extraction methods (*method 1* and *method 2*) was compared. The CE-LIF method was applied to the same baby food FRUIT2 extracted with the *method 1* (see Figure 1.C). In contrast to *method 2*, no peak was observed in the region assigned for βLG peak ($tm_{\beta LG}/tm_{I.S.}$ about 1.046) when employing *method 1* for sample preparation. Only a small shoulder with $tm/t_{I.S.}$ = 1.056 (marked as * in Figure 1.C) was observed between the peak of RhB and the large peak. This result indicates that *method 2* provides larger sensitivity for detecting the allergen than the classical sample preparation method, *method 1*. The innovative sample preparation method is suitable for identifying by CE-LIF the presence of βLG in this baby food formulated with fruit and cereal and contaminated with yoghurt with pear dessert in a ratio corresponding to one part of yoghurt by 1 000 parts of baby food.

The higher effectiveness of *method 2* than of *method 1* for extracting βLG is most probably due to the temperature and to the composition of the extraction solution. The extraction solution in

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method 2 contains β-mercapthoethanol which acts as a disulphide group reducing agent allowing in this way to extract the βLG which could have been incorporated to casein micelles [25] or to other matrix components. The guanidine hydrochloride disrupts the protein conformation making specific regions of the poplypeptide chain more accessible to external reagents. This reagent has proved to be effective for extracting other proteins with different characteristics from other types of foods [26]. The borate buffer acts as a pH regulator. Finally, large salt concentration is used as it enhances the solubility of βLG at the working pH [27].

Estimation of the βLG content

Although the aim of the work was not the quantitation of βLG in these samples but the detection of low levels of the allergen, the developed method made it possible to estimate the concentration of βLG in baby food which is extracted and fluorescently labeled. Using peak height, a linear calibration curve was obtained for standard βLG in the range 5×10^{-10} to 10^{-7} M (correlation coefficient 0.9976). For practical reasons, the standard curve was not obtained by adding βLG to the matrix (sample FRUIT0) because this sample guaranteed to be milk-free is not commercially available to the rest of researchers. The βLG content found in the sample FRUIT2 was 8.0×10^{-9} M (RSD = 7.52 %). This result is in good agreement with the value (1.1×10^{-8} M) obtained by ELISA of the same sample (Table 3), which probably measures not only βLG but also its antigenic peptides [17].

The absence of certified samples of baby food contaminated with dairy desserts containing a known amount of βLG precludes establishing the accuracy of the method of analysis. Besides, the added amount of βLG in the on-purpose contaminated sample is unknown. For sample, FRUIT2 the content of βLG in the yoghurt is not known as addition of whey to yoghurt is an approved practice and also because it depends on the concentration of this protein remaining in the yoghurt after the fermentation process. In addition, as shown below, the accuracy calculated by spiking the sample

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guaranteed to be free of milk with known amounts of standard βLG should not necessarily correspond to the recovery values for samples in which the baby food has been contaminated with the βLG added as dairy dessert and heated afterwards.

To study the influence of the matrix and the thermal treatment on the recovery of βLG in samples containing dairy products several extracted fractions, described in Table 5, were compared. These samples differ in the matrix that was in contact with the dairy product when heating and extraction of βLG were performed. The concentration of βLG detected in these samples, calculated through the peak height in the electropherograms, increased in the order sample A < sample B < sample C. These results seem to indicate that heating the dairy product in the presence of the fruit and cereal baby food matrix favors the βLG interaction with other components of the matrix making more difficult the extraction of this protein. They also seem to indicate that βLG extraction is hampered by the presence of the baby food matrix during the extraction process. Thus, as above indicated, accuracy of the method cannot be calculated by spiking the samples with standard βLG.

Detectability of βLG in samples with lower content of the allergen

The feasibility of the analytical method, including the sample preparation step performed by *method 2*, to detect even lower level of βLG adulterations was checked. To do so, the sample FRUIT3 consisting on the same fruit and cereal matrix than sample FRUIT2 but contaminated on purpose with a smaller amount of dairy product, and submitted to the same thermal process, was analyzed. Figure 1.D shows the electropherogram corresponding to this sample, in which a peak with relative migration time corresponding to βLG (see Table 4) was observed. The βLG content corresponding to this peak in FRUIT3 was 1.3×10^{-9} M (RSD = 7.83 %), this is about one order of magnitude lower than in sample FRUIT2 (8.0×10^{-9} M), which had been contaminated with an amount of dairy dessert 10 times higher (see Table 1). According to this result, the method would be valid to detect the presence of the allergenic protein in the baby food formulated with fruit and

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cereals and that would had suffered contamination during processing even at levels as low as one part of yoghurt in 10 000 parts of baby food.

Applicability of the method to other types of food samples

In order to prove if the described analysis method including *method 2* for sample preparation was applicable to the analysis by CE-LIF of other types of baby foods formulated with different matrices and contaminated by different dairy desserts, baby food samples type FISH (formulated with fish and rice) and MEAT (formulated with chicken meat and rice) were analyzed (see Table 1). Samples of type FISH were contaminated on purpose with a dessert of fresh cheese with fruit. Samples of type MEAT were contaminated with rice pudding made with milk. Contaminations were performed to add a ratio 1:1 000 of dairy product: baby food. Figure 2 shows the electropherograms for these samples (FISH2 and MEAT2) (Fig. 2.A and 2.C) and for the same baby foods matrices guaranteed to be free of βLG (FISH0 and MEAT0) (Fig. 2.B and 2.D). The electrophoretic profiles of both samples of type FISH showed a peak (peak a in Fig. 2 A) with migration time relative to rhodamine 1.080, while a peak with relative migration time corresponding to βLG ($tm_{\beta LG}/tm_{I.S.}$ = 1.046) was only observed in the sample contaminated with the cheese dessert (Table 4). Figures 2.C and 2.D B show the electropherograms for samples MEAT2 and MEAT0. For both samples a peak with relative migration time $tm/tm_{I.S.}$ = 1.057 was observed (peak b in Fig. 2 B). A peak with relative migration time corresponding to βLG ($tm_{\beta LG}/tm_{I.S.}$ = 1.047) was only observed in sample MEAT2 (Table 4). To discard an incorrect assignment of the peak for βLG in MEAT2 due to the closeness of peak b, an immunorecognition assay following the steps previously described was carried out. As expected, the peak with $tm/tm_{I.S.}$ = 1.047 disappeared when the sample had been incubated in the presence of the antibody reinforcing the assignment of this peak to βLG (results not shown). This result was confirmed by spiking the extract of MEAT2 with standard βLG (Fig. 3). It is clearly seen that the It was observed (data not shown) that the height of the peak assigned to βLG increases when increasing the concentration of

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βLG added, confirming the peak identification. By confirming this peak identification the usefulness of RhB as internal standard was reinforced and an interference of the components of baby food types FISH and MEAT on βLG detection was also ruled out. Thus, *method 2* was fully compatible with CE-LIF for the detection of βLG in all the samples studied.

To compare *method 1* and *method 2* for baby food formulated with fish or meat, and contaminated with desserts containing cheese or milk, respectively, the samples FISH2 and MEAT2 were also analyzed by CE-LIF after being extracted by *method 1*. Electropherograms showed no peak for βLG in any of the two samples (results not shown). Thus, for the three types of baby foods (FRUIT, FISH and MEAT) contaminated with the desserts containing any of the three dairy products (yoghurt, cheese or milk) and submitted to thermal treatment, the innovative extraction method developed permitted to detect contaminations that were not possible to be detected when using *method 1* as the sample preparation method.

The amount of βLG extracted by *method 2* from the samples of types MEAT and FISH was calculated from the height of the peaks assigned to this protein in the corresponding electropherograms. The βLG content found in FISH2 and MEAT2 was 3.2×10^{-8} M (RSD = 7.41 %) and 7.6×10^{-9} M (RSD = 8.32 %), respectively. As it was observed for the samples of type FRUIT, also for the FISH and MEAT types the results obtained by CE-LIF are in agreement with those obtained by ELISA for the same samples (see Table 3). The absence of the peak corresponding to βLG in FISH0 and MEAT0 indicates that the concentration of βLG extracted from these sample was lower than the LOD (LOD = 5×10^{-10} M).

4. Concluding remarks

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The new sample preparation method developed for extracting βLG is adequate and fully compatible with CE-LIF to analyze this allergenic protein. The extraction solution containing a disrupting agent, a reducing compound, and large saline concentration applied at 80 ºC allows performing sample preparation in 50 min.

The analysis method, including the innovative sample preparation step and the use of rhodamine B as internal standard, has proved to be valid to detect βLG in baby foods that had been contaminated on purpose simulating the incorporation of dairy products and the further thermal treatment that could take place due to potential contamination in the manufacturing line. Performing spiking with standard βLG and immunorecognition has corroborated the identification of the βLG peak.

The method is valid for different types of baby foods contaminated with desserts formulated with different dairy products. For each of these three types of samples analyzed the new sample preparation method makes possible to detect contaminations by βLG that are undetected when using the classical method for sample preparation. Levels of contamination as low as one part of yoghurt in 10 000 parts of baby food have been detected.

The developed method should be useful in quality control of baby food samples for confirming the absence of βLG as an indicator of contamination by dairy products during the manufacturing process. Furthermore, the described method might have a wide applicability for detecting βLG in any other types of food regardless of the origin of its presence. It could even be useful to detect this allergen in other kind of samples. Thus, the results obtained are promising in the research related to the worldwide problem of cow's milk allergy.

Acknowledgements

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Figures

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Fig. 1. CE-LIF analysis of extracts prepared by two different methods of samples of type FRUIT contaminated with different levels of dairy dessert: (A) FRUIT2 (orange and banana with cereal baby food contaminated (260:1) with yoghurt with pear dessert and extracted by *method 2;* (B) FRUIT0 (orange and banana with cereal baby food guaranteed free of dairy products) extracted with *method 2*; (C) FRUIT2 extracted with *method 1*; (D) FRUIT3 (orange and banana with cereal baby food contaminated (2 600:1) with yoghurt with pear dessert and extracted with *method 2*. CE-LIF conditions: running buffer 6 mM borax-6 mM SDS at pH 9.0, uncoated capillary (L = 60 cm, l = 50 cm, 50 μm I.D.), injection by gravity (h = 20 cm, 12 s) of a mixture of the sample extract with a 10 mM KCN solution and a 10^{-7} M RhB solution followed by an injection of 5 mM FQ in running buffer, mixing time 6 min at 3 kV, reaction time 15 s at 0 kV, mixing and reaction temperature 65 ºC, separation performed at 15 kV and 24 ºC. Peak identification: I.S.: Internal standard rhodamine B, * peak with relative migration time = 1.056.

Fig. 2. CE-LIF analysis of extracts of samples of type FISH and MEAT contaminated and not-contaminated with dairy dessert: (A) FISH2 (hake with rice baby food contaminated (400:1) with fresh cheese with fruit dessert)*;* (B) and FISH0 (hake with rice baby food guaranteed free of dairy products); (C) (B) MEAT2 (chicken with rice baby food contaminated (650:1) with rice pudding); (D) and MEAT0 (chicken with rice baby food guaranteed free of dairy products).All the samples were extracted following *method 2*. CE-LIF conditions as in Figure 1. Peaks a and b as indicated in Table 4. Other peak identification as in Figure 1.

Fig. 3. CE-LIF analysis of: (A) the extract of MEAT2 (chicken with rice baby food contaminated (650:1) with rice pudding); (B) the extracts of MEAT2 spiked with 1×10^{-8} M βLG; (C) the extract of MEAT2 spiked with 5×10^{-8} M βLG. All the samples were extracted following *method 2*. CE-LIF conditions and peaks identification as in Figure 2.

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Table 1
Composition of the samples studied.

Food matrix	Dairy dessert	Dairy dessert : Food matrix ratio	Dairy product: Food matrix ratio[a]	Sample name[b]
Orange and banana with cereal	Yoghurt with pear	Guaranteed free of dairy product	0	FRUIT0
Orange and banana with cereal	Yoghurt with pear	1:26	1:100	FRUIT1
Orange and banana with cereal	Yoghurt with pear	1:260	1:1 000	FRUIT2
Orange and banana with cereal	Yoghurt with pear	1:2 600	1:10 000	FRUIT3
Orange and banana with cereal	Yoghurt with pear	1:26 000	1:100 000	FRUIT4
Hake with rice	Fresh cheese dessert with fruit	Guaranteed free of dairy product	0	FISH0
Hake with rice	Fresh cheese dessert with fruit	1:40	1:100	FISH1
Hake with rice	Fresh cheese dessert with fruit	1:400	1:1 000	FISH2
Hake with rice	Fresh cheese dessert with fruit	1:4 000	1:10 000	FISH3
Hake with rice	Fresh cheese dessert with fruit	1:40 000	1:100 000	FISH4
Chicken with rice	Rice pudding	Guaranteed free of dairy product	0	MEAT0
Chicken with rice	Rice pudding	1:65	1:100	MEAT1
Chicken with rice	Rice pudding	1:650	1:1 000	MEAT2
Chicken with rice	Rice pudding	1:6 500	1:10 000	MEAT3
Chicken with rice	Rice pudding	1:65 000	1:100 000	MEAT4

[a] Ratios calculated knowing that the yoghurt with pear contains 26% of yoghurt, the fresh cheese with fruit contains 40% of cheese, and the rice pudding contains 65% of milk.
[b] Samples of type FRUIT were heated at 105 ºC for 10 min, samples of type FISH were heated at 123 ºC for 45 min, and samples of type MEAT were heated at 121 ºC for 50 min.

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Table 2
Schemes of the sample preparation methods

Method 1	Method 2*
1 g of sample + 10 mL H_2O	1 g of sample + 10 mL (PBS × 10)
↓ Stirr (15 min)	↓ Remove fat → Skimming *vs* non-skimming
↓ Filter (Whatman 40)	↓ Add (β-ME + guanidine hydrochloride + borate) → Simultaneous *vs* sequential addition
↓ Add HCl to pH 4.6	↓ Shake (T^a_1, t_1) → Temperature and time
↓ Left to stand (room T^a, 20 min)	↓ Add NaCl → Simultaneous *vs* sequential addition
↓ Centrifuge (4500 g, 20 min, 4 ºC)	↓ Shake (T^a_2, t_2) → Temperature and time
↓ Collect supernatant	↓ Centrifuge (9000g, 35 min, room T^a)
↓ Filter (PVDF, 0.45 µm)	↓ Filter (Whatman 40)
↓ Filter (Tuffryn, 0.22 µm)	↓ Filter (PVDF, 0.45 µm)

* The parameters shown in boxes have been optimized in the present work.

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Table 3
Concentration of βLG detected on the samples studied depending on the sample preparation method (*method 1 vs method 2*) and the analysis method (ELISA *vs* CE-LIF) employed.

SAMPLE	DETECTED βLG (n=4)		
	Method 1	*Method 2*	
	ELISA	ELISA	CE-LIF
	Mean (M)	Mean (M)	Mean (M)
FRUIT1	4.0×10^{-9}	6.5×10^{-7}	N.A.
FRUIT2	b	1.1×10^{-8}	8.0×10^{-9}
FRUIT3	a	3.2×10^{-9}	1.3×10^{-9}
FRUIT4	a	2.1×10^{-9}	N.A.
FISH1	1.0×10^{-7}	5.2×10^{-7}	N.A.
FISH2	1.1×10^{-8}	2.1×10^{-8}	3.2×10^{-8}
FISH3	b	4.4×10^{-9}	N.A.
FISH4	b	2.4×10^{-9}	N.A.
MEAT1	6.9×10^{-9}	5.1×10^{-8}	N.A.
MEAT2	2.5×10^{-9}	8.4×10^{-9}	7.6×10^{-9}
MEAT3	a	a	N.A.
MEAT4	a	a	N.A.

a: Absorbance value similar to the blank assay.
b: Absorbance value lower than the limit of quantitation (2×10^{-9} M) but higher than the blank assay.
N.A.: Not analyzed.

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Table 4
CE-LIF migration time (tm) and migration time relative to the I.S. ($tm/tm_{I.S.}$) for the peaks of interest.

Sample	Number of injections	$tm_{I.S.}$		$tm_{\beta LG}$		$tm_{\beta LG}/tm_{I.S.}$		$tm_{peak\ a}$		$tm_{peak\ a}/tm_{I.S.}$		$tm_{peak\ b}$		$tm_{peak\ b}/tm_{I.S.}$	
		Mean (min)	RSD (%)	Mean (min)	RSD (%)	Mean	RSD (%)	Mean (min)	RSD (%)	Mean (min)	RSD (%)	Mean	RSD (%)	Mean	RSD (%)
FRUIT2	20	8.793	1.09	9.194	1.02	1.046	0.12	n.o.		n.o.		n.o.		n.o.	
FRUIT3	7	8.774	1.45	9.197	1.41	1.048	0.08	n.o.		n.o.		n.o.		n.o.	
FRUIT0	8	9.694	0.32	n.o.		n.o.		n.o.		n.o.		n.o.		n.o.	
FISH2	6	8.562	1.61	8.957	1.60	1.046	0.07	9.240	1.81	1.079	0.22	n.o.		n.o.	
FISH0	6	8.517	0.51	n.o.		n.o.		9.195	0.57	1.080	0.15	n.o.		n.o.	
MEAT2	7	8.643	1.47	9.050	1.52	1.047	0.12	n.o.		n.o.		9.134	1.52	1.057	0.13
MEAT0	7	8.561	0.57	n.o.		n.o.		n.o.		n.o.		9.053	0.53	1.057	0.14

$tm_{I.S.}$: migration time of RhB peak; $tm_{\beta LG}$: migration time of βLG peak; $tm_{\beta LG}/tm_{I.S.}$: migration time of βLG peak relative to migration time of RhB; $tm_{peak\ a}$: migration time of peak a; $tm_{peak\ a}/tm_{I.S.}$: migration time of peak a relative to migration time of RhB; $tm_{peak\ b}$: migration time of peak b; $tm_{peak\ b}/tm_{I.S.}$: migration time of peak b relative to migration time of RhB.
Peaks a and b are shown in Fig. 2 and 3.
n.o.: Not observed.

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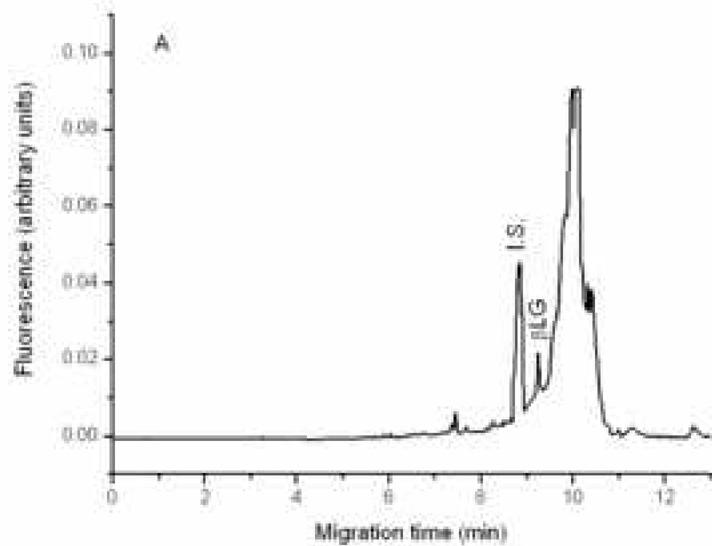
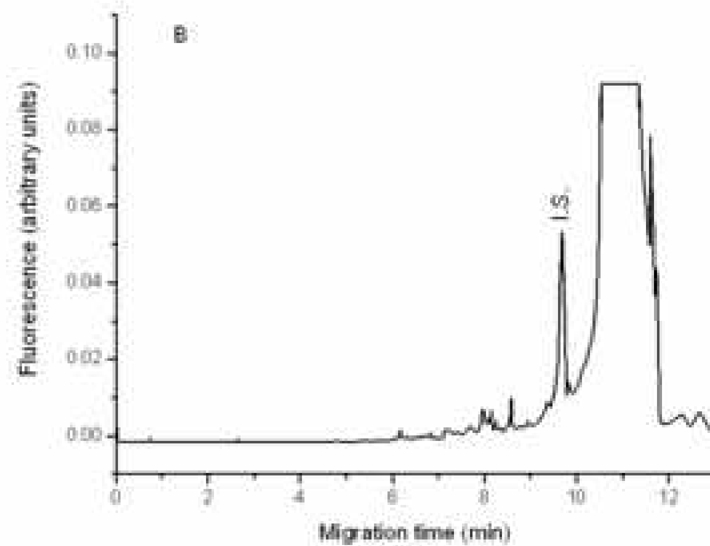
Table 5
Influence of the matrix and of the thermal treatment on the concentration of βLG detected by CE-LIF in samples containing dairy products.

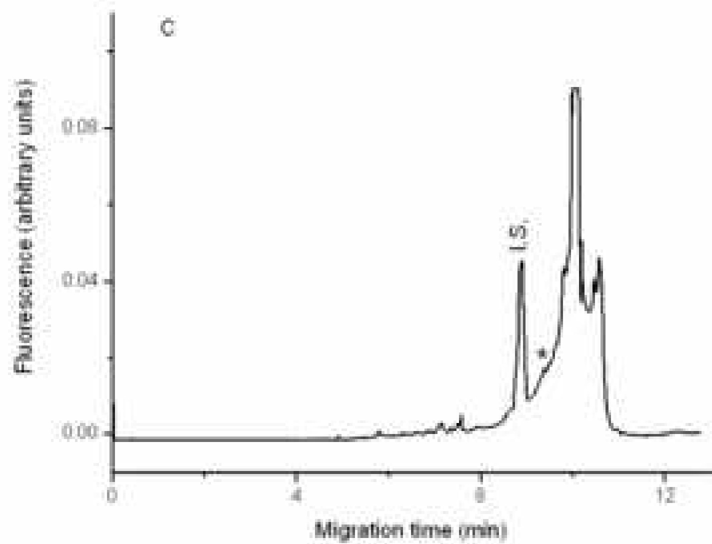
SAMPLE	DESCRIPTION OF THE PROCESS*	SCHEMATIC PROCESS	DETECTED βLG
Sample A (Extract of FRUIT2)	Extraction of the orange and banana with cereal baby food contaminated (260:1) with yoghurt with pear.	Baby food guaranteed milk free (FRUIT0) ↓ Addition of YOGHURT WITH PEAR (260:1 ratio) ↓Heating (100 ºC, 30 min) ↓βLG extraction SAMPLE A	8.0×10^{-9} M
Sample B	Extraction of mixture of the heated orange and banana with cereal baby food βLG free and the heated yoghurt with pear.	Baby food guaranteed milk free / YOGHURT WITH PEAR ↓Heating (100 ºC, 30 min) / ↓Heating (100 ºC, 30 min) B1 / B2 ↓ ↓ Mixture B1:B2 (ratio 260:1) ↓ βLG extraction SAMPLE B	1.2×10^{-8} M
Sample C	Mixture of the extract of the heated orange and banana with cereal baby food βLG free and the extract of the heated yoghurt with pear.	Baby food guaranteed milk free / YOGHURT WITH PEAR ↓Heating (100 ºC, 30 min) / ↓ Heating (100 ºC, 30 min) ↓βLG extraction / ↓βLG extraction C1 / C2 ↓ ↓ Mixture C1:C2 (ratio 260:1) ↓ SAMPLE C	3.0×10^{-8} M

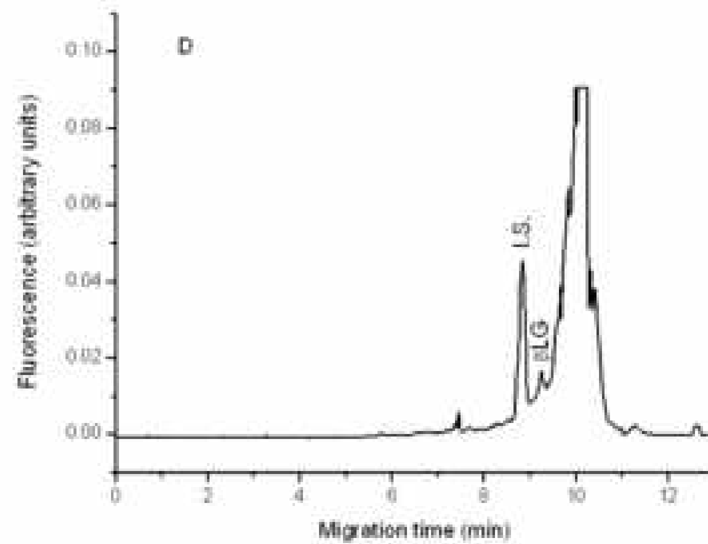
* All the extractions were performed by *method 2*.

8. Figure 1









8. Figure 2