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Development of an Optimized ELISA and a Sample Preparation Method for the Detection of β-Lactoglobulin Traces in Baby Foods

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Dairy products can induce allergic reactions even when present at very low levels, such as levels found in involuntary contamination during food manufacturing. β-Lactoglobulin (βLG) is the main allergen in cow's milk. The objective of this work was to develop a sensitive method for βLG detection in baby foods through the optimization of an innovative sample preparation method. Three types of baby foods deliberately contaminated with dairy products or dairy desserts were sterilized to simulate the potential contamination occurring during manufacturing and then used as samples. Different sample preparation methods were compared. The best results were provided by an extraction solution containing β-mercaptoethanol, guanidine hydrochloride, and a saline solution. An ELISA method was optimized for the detection of βLG (LOD = 9.7×10^{-13} M). The developed method allowed detection of even 1 part of dairy product in 100,000 parts of baby food for some of the analyzed foods.

KEYWORDS: β-Lactoglobulin; trace levels; allergen; baby food; sample preparation method; ELISA

INTRODUCTION

The best nutritional option for newborn infants is breast feeding. However, from the sixth month of life onward, pediatricians suggest that breast milk should be replaced by complementary food (*1*). At this age, most infants begin to follow diets with additional ingredients, and commercial baby foods play an important role in their nutrition. Despite the high nutritional value of baby foods, the introduction of additional ingredients in the baby's diet might result in immunologic reactions.

Food-related allergies affect up to 6% of young children (*2*) and have become one of the main research topics for infant health. The immunologic reaction to milk proteins is considered to be the most common food allergy. Milk is a key ingredient in a wide variety of baby foods to ensure the nutritional requirements for infants are met. Many food industries use the same machinery to produce dairy and nondairy food and, therefore, a risk of cross-contamination exists. Some milk-allergic individuals, especially infants, are sensitive even to traces of cow's milk proteins in commercial nondairy foods. In these highly sensitized individuals, the intake of trace amounts of β-lactoglobulin (βLG) can induce digestive disorders (diarrhea), respiratory symptoms (allergic asthma, rhinoconjunctivitis, chronic cough), and skin reactions (urticaria, atopic dermatitis) (*3*). At the present time, no effective preventive treatments exist for milk allergy. Hence, susceptible individuals are required to strictly avoid foods containing milk within their diet. Consequently, it is important to be able to obtain food products that are guaranteed to be free of dairy ingredients.

The seriousness of food allergies led the European Commission to regulate the labeling of main food allergens. According to the latest European Union Labeling Directive (Directive 2007/68/ED 27.11.2007), milk and its derivatives must be labeled on commercial foods (*4*).

Accordingly, food industries require the use of accurate and sensitive detection methods for food allergens to control better their production processes and to ensure the correct labeling of their products in the effort to protect sensitive consumers.

Bovine βLG synthesized in the mammary gland of ruminants is the dominant whey protein (58% w/w) (*5*). It has a molecular mass of about 18 kDa, and it contains two intramolecular disulfide bonds and one residue of cysteine per monomer. The cysteine is especially important because it reacts, following heat denaturation, with the molecular disulfide of κ-casein and affects the availability of free βLG (*6*). Although βLG is not the only allergenic component in cow's milk, it is one of the major allergenic proteins and, for this reason, is the focus of the present study.

The use of very highly sensitive detection techniques is necessary to detect allergens. Several methods have been reported for the detection of dairy products in food. The most popular are antibody-based techniques, mainly in the configurations of enzyme-linked immunosorbent assays (ELISA) and biosensors (*7*). The present study uses ELISA for the detection of βLG as it is sensitive, specific, and simple and allows the analysis of several samples simultaneously, which is desirable in routine screening.

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It is well-known that the detection of proteins in samples usually requires a prior sample preparation step that enables protein extraction from the food matrix (*7*−*9*). Total protein extraction from processed foods is not always an easy task. In many cases a very small amount of the protein, in this case βLG, is present in the food source. Additionally, protein extraction is further complicated due to the use of food matrices that are formed by different compounds such as fats, oils, phenols, or carbohydrates with which the protein can interact. In the past, several methods have been described for the isolation of βLG from milk (*10*). The usual method for this allergen isolation from milk is the protocol set up by Bleumink and Young in 1968 (*11*). By following this protocol caseins were separated from the whey fraction, which contained soluble nonbound βLG, by precipitation with 1 M HCl at pH 4.65.

Different commercial immunoassay kits recommend the use of either this type of acidic solution, water, or a saline solution to extract this allergen prior to performance of the immunoassay. However, the efficiency of these methods for extracting βLG from temperature-processed foods based on complex matrices contaminated with low amounts of dairy products has not been studied. The absence of certified materials containing known amounts of dairy products in processed foods hinders this type of study. Performing studies in food spiked with standard βLG does not necessarily reflect the behavior in milk-contaminated processed food because in spiked samples the interactions of βLG with itself and with other components of the food, especially with κ-casein (κCN), caused by heating do not take place. It has been demonstrated that even in model systems (in which heating of βLG and κCN was performed in the absence of milk matrix) the disulfide-bonded products were different from those formed in milk (*12*).

In this work different methods for extracting βLG are compared. Evaluation is performed using an optimized ELISA. Samples of baby food based on chicken, fish, and fruit matrices were elaborated and were deliberately contaminated with known amounts of dairy products and with dairy desserts containing dairy products. Contaminations were performed to simulate the cross-contamination that potentially could happen during food manufacturing.

MATERIALS AND METHODS

Chemicals and Devices. Sodium tetraborate decahydrate (borax), guanidine hydrochloride (GdnHCl), potassium chloride, β-mercaptoethanol (βME), *o*-phenylenediamine (OPD), rabbit serum (type S, without thimerosal, and type R, which contains thimerosal), ovalbumin, and standard βLG (A+B, crystallized, 90% purity, L-0130) were purchased from Sigma (St. Louis, MO). Disodium hydrogenphosphate, sodium chloride, sodium dihydrogenphosphate, sodium hydroxide, polyoxyethylene sorbitan monolaurate (Tween 20), and 3-(lauryldimethylammonio)-propane-1-sulfonate (Zwittergent) were from Merck (Darmstadt, Germany). Hydrochloric acid, sulfuric acid, and sodium carbonate were from Panreac (Barcelona, Spain). Boric acid was from Fluka (Buchs, Switzerland). Concentrated buffer with stabilized hydrogen peroxide for enzymatic reaction with horseradish peroxidase was purchased from Pierce (Rockford, IL). Affinity-purified anti-bovine βLG (A+B) raised in rabbit (antiβLG) unconjugated and conjugated with horseradish peroxidase (antiβLG-HRP) were purchased from Bethyl Laboratories (Montgomery, TX). Water from a Milli-Q water system (Millipore, Bedford, MA) was used throughout.

OPD solution (0.5 mg/mL), substrate for HRP, was prepared every day in buffer containing stabilized H_2O_2. This buffer was prepared in the moment of use from the 10× concentrated commercial solution.

βLG aqueous standard solutions in the range 1×10^{-7}−5×10^{-10} M were prepared from a 1 mg/mL βLG stock solution, which was filtered through a low protein binding filter of 0.22 μm pore size (Millipore). The aqueous stock solution was aliquoted in commercial low-binding polymer vials (Sorenson BioScience, Salt Lake City, UT) and stored at −4 °C. Every working day an aliquot was thawed, and at the end of the day it was discarded.

Microtiter plates (NUNC-Immuno MaxiSorp 96-MicroWell plates, NUNC, Rochester, NY) with C bottom shape were used to perform the ELISAs.

Food Samples. A group of 28 samples was analyzed. The set included heat-treated milk, 3 baby foods guaranteed free of dairy products, and 24 baby food samples deliberately contaminated with dairy products and with dairy desserts. Proportions of dairy products and dairy desserts, with their corresponding content in dairy products, added to each of the samples are indicated in **Table 1**. The baby food samples analyzed were of three types of matrices: meat, fish, and fruit. The main components in these matrices were chicken with rice in type meat, hake with rice in type fish, and orange and banana with cereal in type fruit. Samples named 1A, 2A, and 3A corresponded to baby foods of each type guaranteed to be free of dairy products. These milk-free baby food samples were provided by Hero España S.A., Murcia, Spain. These samples were prepared by mixing the ingredients on a laboratory scale (milk-free conditions) and processing them under industrial conditions at 121 °C for 50 min for meat-type samples, at 123 °C for 45 min for fish-type samples, and at 105 °C for 10 min for fruit-type samples. These matrices were deliberately contaminated either with known amounts of previously processed dairy products or with dairy desserts by mixing them on a laboratory scale and processing them under industrial conditions at 121 °C for 50 min for meat-type samples, at 123 °C for 45 min for fish-type samples, and at 105 °C for 10 min for fruit-type samples, to simulate the cross-contamination that could happen during the manufacturing process. The dairy products were powdered milk (1 g of powdered milk was suspended in 6.64 mL of water), fresh cheese (which had been pasteurized by the supplier), and yogurt (also pasteurized by the supplier) for the sample types meat, fish, and fruit, respectively. The dairy desserts used to contaminate the matrices were rice pudding, fresh cheese dessert with fruit, and yogurt with pear for the sample types meat, fish, and fruit, respectively. The rice pudding contained 65% milk. The fresh cheese with fruit had 40% fresh cheese. The yogurt with pear contained 26% yoghurt. Also, the milk used to contaminate the meat-type samples was submitted to the thermal treatment of the corresponding manufacturing line, that is, 50 min at 121 °C.

Optimization of the ELISA Method: Parameters Studied. We have developed a sandwich-type ELISA. For the optimization of the method previous works from our group (*13*, *14*) and other author guidelines (*15*, *16*) were taken into account. In every assay some plate wells were used for the blank assays, in which samples, antibodies, or substrates of enzymatic reaction were substituted for the corresponding solvents. The sandwich ELISA was developed by utilizing polyclonal antiβLG raised in rabbit as capture antibody and the same antibody conjugated to horseradish peroxidase as labeled antibody. The general method was as follows: Plates were coated overnight for each well at 4 °C with 100 μL of capture antibody diluted in 50 mM sodium carbonate/bicarbonate buffer, pH 9.6. After the solution had been discarded, the wells were cleaned with washing solution and passivated with 200 μL of the passivating solution for 1 h at room temperature. The plates were cleaned again with washing solution and incubated for 1 h at room temperature with 100 μL of standard βLG or sample extract in dilution solution. Afterward, washing was performed with water. One hundred microliters of the conjugated antibody in dilution solution was added to each well, and the plates were incubated for 1 h at room temperature. The plates were cleaned with washing solution and rinsed with Milli-Q water. After rinsing, 100 μL of the substrate was added to each well. The enzymatic reaction was stopped by adding 50 μL of 2.5 mM sulfuric acid in each well. The color produced was measured at 492 nm in a microplate reader (Molecular Devices, Sunnyvale, CA). Standard curves were generated using version 3.1.2 of SoftMax Pro software (Molecular Devices).

For the optimization of the ELISA the influence of the following variables was studied: *dilutions* of the commercial solutions of the capture antibody in the range from 1:10000 to 1:1000 and of the conjugated antibody in the range from 1:100000 to 1:100 were assayed; with regard to the *composition of the washing and dilution solutions*, several additives in different concentrations, such as surfactants (0.0001−0.1% Zwittergent, 0.05% Tween 20), proteins (0.0002−0.2% ovalbumin), and two different rabbit serums (rabbit serum type S and type R) at two different concentrations,

Table 1. Baby Food Samples Studied and Comparison of Concentration of βLG Detected by the Optimized ELISA Method in Samples Prepared following the Classical Method (Procedure 1) and the One Optimized in This Work (Procedure 7)[a]

food matrix	dairy product or dairy dessert added	dairy dessert/food matrix ratio	dairy product/food matrix ratio[b]	sample code	detected βLG (μg/g) procedure 1,[c] mean ± SD	detected βLG (μg/g) procedure 7,[c] mean ± SD
chicken with rice		guaranteed free of dairy product	0	1A	a	a
chicken with rice	milk		1:100	1E1	0.059 ± 0.009	0.182 ± 0.011
chicken with rice	milk		1:1000	1E2	NA	0.056 ± 0.005
chicken with rice	milk		1:10000	1E3	NA	b
chicken with rice	milk		1:100000	1E4	NA	b
chicken with rice	rice pudding	1:65	1:100	1D1	0.041	0.126 ± 0.011
chicken with rice	rice pudding	1:650	1:1000	1D2	NA	0.045 ± 0.005
chicken with rice	rice pudding	1:6500	1:10000	1D3	NA	a
chicken with rice	rice pudding	1:65000	1:100000	1D4	NA	a
hake with rice		guaranteed free of dairy product	0	2A	a	a
hake with rice	fresh cheese		1:100	2E1	1.510 ± 0.250	3 ± 0.198
hake with rice	fresh cheese		1:1000	2E2	NA	0.240 ± 0.013
hake with rice	fresh cheese		1:10000	2E3	NA	0.047 ± 0.010
hake with rice	fresh cheese		1:100000	2E4	NA	b
hake with rice	fresh cheese dessert with fruit	1:40	1:100	2D1	0.086 ± 0.013	1.880 ± 0.176
hake with rice	fresh cheese dessert with fruit	1:400	1:1000	2D2	NA	0.198 ± 0.023
hake with rice	fresh cheese dessert with fruit	1:4000	1:10000	2D3	NA	b
hake with rice	fresh cheese dessert with fruit	1:40000	1:100000	2D4	NA	b
orange and banana with cereal		guaranteed free of dairy product	0	3A	a	a
orange and banana with cereal	yogurt		1:100	3E1	a	0.048 ± 0.026
orange and banana with cereal	yogurt		1:1000	3E2	NA	b
orange and banana with cereal	yogurt		1:10000	3E3	NA	a
orange and banana with cereal	yogurt		1:100000	3E4	NA	a
orange and banana with cereal	yogurt with pear	1:26	1:100	3D1	a	0.074 ± 0.005
orange and banana with cereal	yogurt with pear	1:260	1:1000	3D2	NA	b
orange and banana with cereal	yogurt with pear	1:2600	1:10000	3D3	NA	a
orange and banana with cereal	yogurt with pear	1:26000	1:100000	3D4	NA	a

[a] Proportions of dairy products and dairy desserts in the infant food samples are indicated. [b] Ratios calculated knowing that the rice pudding contains 65% milk, the fresh cheese dessert contains 40% fresh cheese, and the yogurt with pear contains 26% yogurt. [c] NA, not analyzed; a, absorbance value similar to the blank assay; b, absorbance value lower than the limit of quantification (0.036 μg/g = 2×10^{-9} M) but higher than the blank assay.

0.1−0.5%, were tested to avoid nonspecific adsorption of compounds in the plate; with regard to the *composition of the passivating solution*, phosphate buffer saline (PBS; 0.01 M phosphate buffer, 0.137 M NaCl, 0.0027 M KCl, pH 7.4) solution with addition of both types of rabbit sera at concentrations of 0.1 and 0.5% were assayed; serial dilutions of standard βLG and sample extracts were performed either in glass tubes, prior to addition to the plate, or in the plate itself *to test the effect of protein adsorption on the vessel*; the effect of either *covering the plates* with Parafilm paper or introducing them inside a humid chamber during incubation steps was studied; *the reaction time between the substrate and the enzyme was selected* by measuring the absorbance obtained as a result of the reaction every 15 s, without stopping the reaction with sulfuric acid. The incubation time range studied was 0−120 min. The optimal incubation time was determined by the slope of the curve absorbance versus incubation time. For this assay, absorbance was measured at 450 nm.

Sample Preparation. To optimize the extraction of βLG from the samples, different sample preparation procedures were compared. The effect of several factors, such as nature and concentration of extracting agents, solvent of the sample, centrifugation forces and times, and filtering procedures, was studied. A scheme of each of the seven procedures is shown in **Figure 1**.

In these procedures "the sample" refers to any of the 28 samples mentioned above. Centrifugation was performed using a Biofuge 22R centrifuge (Heraeus, Hanau, Germany). Whatman no. 40 filters were from Whatman International (Maidstone, U.K.), Millex syringe filters PVDF membrane of 0.45 μm pore size were from Millipore, and Tuffryn membrane Acrodisc syringe filters of 0.22 μm pore size were from Pall Corp. (Ann Arbor, MI). In procedures 2−7, 5% (v/v) of 2.5 mM borate buffer at pH 8.3 was added. For all of the procedures, analysis by ELISA was performed within 24 h of sample preparation.

RESULTS AND DISCUSSION

Optimization of the ELISA Method. The effect of the concentration of antiβLG and antiβLG-HRP on the sensitivity of the ELISA was first studied. For this purpose, several curves of standard βLG were obtained in the same ELISA plate with different combinations of the concentrations of antiβLG and antiβLG-HRP. A dilution of 1:1000 of both commercial antibodies, antiβLG and antiβLG-HRP, provided the highest slope in the linear range of the semilogarithmic curve plotting absorbance versus concentration for standard βLG (results not shown); this indicates the highest sensitivity in ELISA detection. For the washing, dilution, and passivation solutions, two different rabbit sera were tested. Because no differences between them were observed, the less expensive one (type R) was chosen. The additives that provided the highest slope for standard βLG and the lowest nonspecific interactions were 0.05% (w/v) Tween 20 together with 0.05% (w/v) rabbit serum for the washing and dilution solutions or 0.5% (w/v) rabbit serum for the passivation solution. When the passivation time was increased from 30 to 60 min, the nonspecific interactions were diminished. As a result of performing the serial dilutions of standard βLG and sample

PROCEDURE 1
1 g of sample + 40 mL H_2O
↓ Remove fat
↓ Stirr (15 min)
↓ Filter (Whatman 40)
↓ Add HCl to pH 4.6
↓ Let to stand (room Tª, 20 min)
↓ Centrifuge (4500 g, 20 min, 4 ºC)
↓ Collect supernatant
↓ Filter (PVDF, 0.45 µm)
↓ Filter (Tuffryn®, 0.22 µm)

PROCEDURE 2
1 g of sample + 40 mL H_2O
↓ Remove fat
↓ Add 30 mL 0.15 M NaCl
↓ Mix + shake (room Tª, 2 h)
↓ Centrifuge (9000 g, 35 min, room Tª)
↓ Collect supernatant
↓ Filter (Whatman 40)
↓ Filter (PVDF, 0.45 µm)

PROCEDURE 3
1 g of sample + 40 mL H_2O
↓ Remove fat
↓ Add (β-ME + borate buffer)
↓ Mix + shake (room Tª, 2 h)
↓ Centrifuge (9000 g, 35 min, room Tª)
↓ Collect supernatant
↓ Filter (Whatman 40)
↓ Filter (PVDF, 0.45 µm)

PROCEDURE 4
1 g of sample + 40 mL H_2O
↓ Remove fat
↓ Add (β-ME + borate buffer)
↓ Mix + shake (room Tª, 2 h)
↓ Add 30 mL 0.15 M NaCl
↓ Mix + Shake (room Tª, 1h)
↓ Centrifuge (9000 g, 35 min, room Tª)
↓ Collect supernatant
↓ Filter (Whatman 40)
↓ Filter (PVDF, 0.45 µm)

PROCEDURE 5
1 g of sample + 40 mL H_2O
↓ Remove fat
↓ Add 30 mL 0.15 M NaCl
↓ Mix + shake (room Tª, 1 h)
↓ Add (β-ME + borate buffer)
↓ Mix + shake (room Tª, 2 h)
↓ Centrifuge (9000 g, 35 min, room Tª)
↓ Collect supernatant
↓ Filter (Whatman 40)
↓ Filter (PVDF, 0.45 µm)

PROCEDURE 6
1 g of sample + 40 mL H_2O
↓ Remove fat
↓ Add (β-ME + GdnHCl + borate buffer)
↓ Mix + shake (room Tª, 2 h)
↓ Add 30 mL 0.15 M NaCl
↓ Mix + shake (room Tª, 1 h)
↓ Centrifuge (9000 g, 35 min, room Tª)
↓ Collect supernatant
↓ Filter (Whatman 40)
↓ Filter (PVDF, 0.45 µm)

PROCEDURE 7
1 g of sample + 40 mL PBS
↓ Remove fat
↓ Add (β-ME + GdnHCl + borate buffer)
↓ Mix + shake (room Tª, 2 h)
↓ Add 30 mL 0.15 M NaCl
↓ Mix + shake (room Tª, 1 h)
↓ Centrifuge (9000 g, 35 min, room Tª)
↓ Collect supernatant
↓ Filter (Whatman 40)
↓ Filter (PVDF, 0.45 µm)

Selected conditions: [β-ME]=24 mM; [GdnHCl] = 25 mM.

Figure 1. Schemes of the sample preparation methods studied.

extracts in the plate itself, the reproducibility of the results and the simplicity of the ELISA method increased noticeably. Incubation under humidity was remarkably essential in the ELISA method to avoid evaporation of the solutions during the incubation periods. The time allowed for the enzymatic reaction to take place had a noticeable effect on the results obtained by ELISA. The absorbance value for the most concentrated standard should be close to 2 and the absorbance value for the most diluted standard close to the value for the blank (*17*). The kinetic studies developed in this work for different antiβLG and antiβLG-HRP concentrations demonstrated that the sensitivity was maximal when the enzymatic reaction was allowed to take place in the range from 100 to 300 s (results not shown). Two minutes was chosen as the time at which the enzymatic reaction was stopped, as indicated in the procedure described under Materials and Methods.

Figure 2 shows a curve for standard βLG in the concentration range from 9.7×10^{-13} to 1.6×10^{-6} M obtained by the ELISA optimized procedure. The practical limit of detection (LOD) of the ELISA method was calculated to be 9.7×10^{-13} M for standard βLG; the absorbance produced by this concentration was always clearly higher than that of a blank. This estimated LOD of this method is between 1 and 3 orders of magnitude lower than the one obtained by other ELISAs described in the literature to determine standard βLG (*16*−*19*). The linearity defined as the capability of the method to provide results that are proportional to the concentration of the analyte in the working range of concentrations (*20*) corresponds in the ELISA optimized to the interval 3.5×10^{-11}−4.6×10^{-8} M for standard βLG, which is a linear range of approximately 3 orders of magnitude. These values correspond to 0.036 and 32.94 μg/g (2.0×10^{-9} and 1.8×10^{-6} M), respectively, of βLG in the samples, taking into account that a dilution of 1:40 takes place in the sample preparation

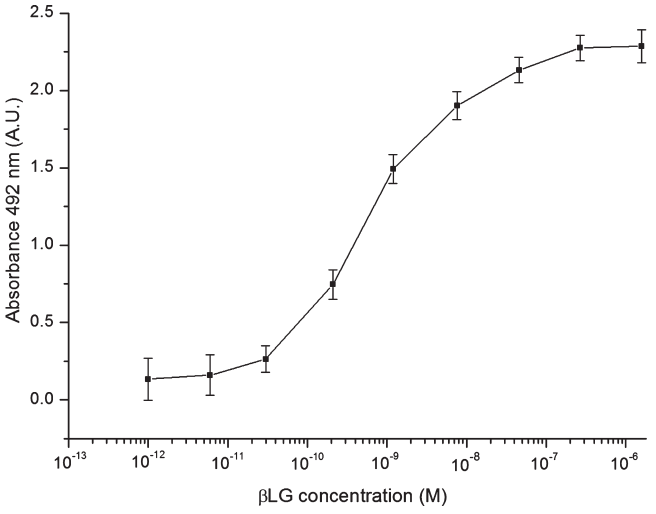


Figure 2. Semilogarithmic curve for standard βLG in the range 9.7×10^{-13}−1.6×10^{-6} M obtained by sandwich ELISA under the optimized conditions. Each point represents the mean of 10 determinations. Vertical bars correspond to ±2 SD values.

procedure. This linear range is 1 order of magnitude (*17*) wider than others previously published for βLG ELISA and 2 orders of magnitude wider than others published for other food allergens (*21*). The calibration curve obtained was absorbance = 7.868 + 0.728 log βLG (M), with a correlation coefficient of 0.986. The lower limit of quantitation (LOQ), defined as the lowest level of analyte in a sample that can be measured with accuracy and precision (*20*), is 3.5×10^{-11} M for standard βLG in the ELISA plate, which corresponds to 0.036 μg/g (2×10^{-9} M) of βLG in the undiluted samples.

Table 2. Influence of the Procedure Used for Sample Preparation[a]

Sample	Concentration of βLG detected (μg/g) Mean ± SD (n=6)						
	Procedure 1	*Procedure 2*	*Procedure 3*	*Procedure 4*	*Procedure 5*	*Procedure 6*	*Procedure 7*
1E1	0.059 ± 0.009	d	d	0.082 ± 0.012	d	0.133 ± 0.007	0.182 ± 0.009
1A	C	c	c	c	c	c	c

[a] Detection by the optimized ELISA method of βLG in meat baby food deliberately contaminated with milk. Sample 1E1 is meat baby food purposely contaminated (100:1) with milk and submitted to 121 °C for 50 min. Sample 1A is meat baby food guaranteed free of milk. [b] Absorbance value lower than the limit of quantification (0.036 μg/g = 2×10^{-9} M) but higher than the blank assay. [c] Absorbance value similar to the blank assay.

Sample Preparation Optimization. Several approaches were investigated to find the most suitable sample preparation method in terms of highest recovery, no interference in the signal provided for βLG by ELISA, and ease of use.

Table 2 shows the ELISA results obtained for sample 1E1 (see **Table 1** for sample denomination), which corresponds to meat baby food deliberately contaminated with 1% milk prepared with each sample preparation procedure. The conventional extraction method (procedure 1) for isolation of whey from dairy products (*22*) was the first approach assayed for the purpose of this work. The separation of the whey and casein fraction is extensively used; however, it is known that during the heat treatment of the processed foods βLG is denatured and binds to casein micelles (*23*, *24*). Cysteine at position 160 (Cys 160) of βLG is implicated in disulfide bond interchange reactions between βLG monomers or polymers and κ-casein (*25*). By heating model solutions of βLG and κ-casein at conditions typical of industrial heat treatments, it has been demonstrated that βLG can form disulfide bonds with both residues C11 and C88 of κ-casein (*24*). The results found by ELISA by Addeo et al. indicate that the amount of βLG−casein aggregates depends on the heat treatment of the milk. For UHT-treated milk the largest percentage (> 90%) of βLG was found associated via disulfide bridges to casein aggregates for bovine mozzarella cheeses. Heat treatment as low as that used in pasteurization produces a marked increase in the percentage of βLG in the fraction insoluble at pH 4.6 in comparison to cheese made of raw milk (*26*).

As a consequence, to determine the βLG in commercial foods, analysis of the protein concentration in both the whey and casein fractions is required. For this reason, some reagents able to extract βLG that could have been incorporated to casein micelles were added to the extraction solution for the simultaneous extraction of βLG from the whey and casein fractions. First, in this study, sodium chloride was assayed as the only extracting agent (procedure 2). Some authors have reported that a high salt concentration is desirable in the extraction solution as it causes the separation of proteins in samples, from sugars, lipids, and phenolic compounds, which often bind to proteins and cause purification problems (*27*). Moreover, large salt concentrations are used to enhance the solubility of βLG at pH 6.5−7 (*28*). However, the results obtained showed that the use of sodium chloride as the only component of the extraction solution (procedure 2) did not provide enough sensitivity for βLG analysis. This behavior could indicate that aqueous saline solvents, such as sodium chloride, are only effective in extracting proteins present in food and not submitted to thermal treatments. For this reason, another method was investigated.

βME has been used for extracting βLG from the caseinic fraction. When βLG is denatured by heating milk, this protein combines with the caseins via disulfide bonding. βME reduces these covalent bonds (*29*, *30*). Thus, βME was assayed as an extracting agent for our purpose. First, the effect on the absorbance provided by βME in ELISA was carefully checked. For this purpose, we studied the highest possible concentration of βME that would not affect the absorbance in the ELISA method. βME concentrations in the range of 5−100 mM were studied. It was observed that a change in the concentration of this reagent did not have a marked influence in the βLG standard curve (**Figure 3**). Thus, a concentration of 24 mM βME was selected for the extracting solution because it had been observed in previous assays to provide good results for βLG extraction.

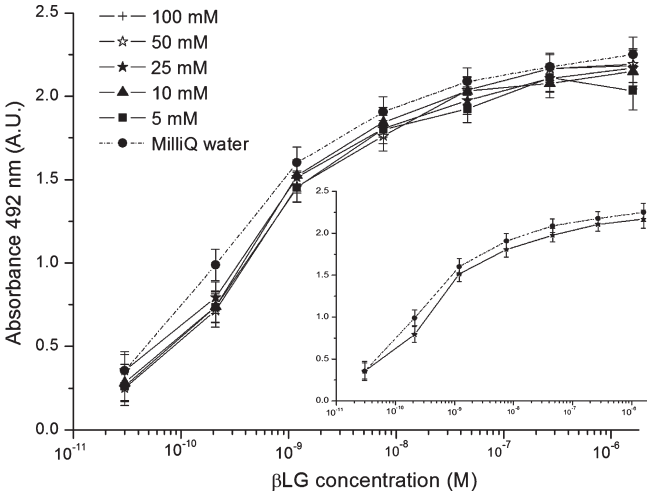


Figure 3. Curve obtained by ELISA under the optimized conditions for aqueous solutions of standard βLG and for standard βLG in PBS with different concentrations of βME in the solvent buffer, indicated by the different symbols in the figure. Assays were performed in triplicate. Vertical bars correspond to ±2 SD values. (Inset) Aqueous solution of standard βLG and standard βLG in PBS with 25 mM βME.

βME was used as an extracting agent in procedure 3. As already observed by other authors a pH of around 7 favors the effectiveness of βME to disrupt the binding of βLG to the casein micelles (*29*). For this reason, a borate buffer was added to βME solution to increase the pH value of the extractant. As a result, the pH value increased by > 1 unit when compared to the pH of the extraction solution before the addition of the borate buffer (pH was 5.5 before the addition of borate and 6.7 after the addition of borate). However, the sensitivity of the method was as low as for procedure 2 (see **Table 2**). When the protein was extracted with βME and borate buffer, and solubilized afterward by the addition of a saline solution (procedure 4), the sensitivity of the method was the highest of the above-mentioned procedures. When the protein was first solubilized in the saline solution and afterward the βME and borate buffer were added (procedure 5), the βLG extraction was negatively affected compared to treatment with the same reagents in the opposite order. It is probable that the large salt concentration enhances the solubility of the protein only once it has been extracted from the casein micelles, which would also justify the lack of solubility observed when only NaCl is employed as extracting agent.

GdnHCl is a disaggregating agent that disrupts the βLG conformation (*31*). The influence of different concentrations (5−200 mM) of GdnHCl in the ELISA signal for βLG was studied. It was observed that for concentrations of ≥50 mM, a decrease in the absorbance curve obtained by ELISA for standard

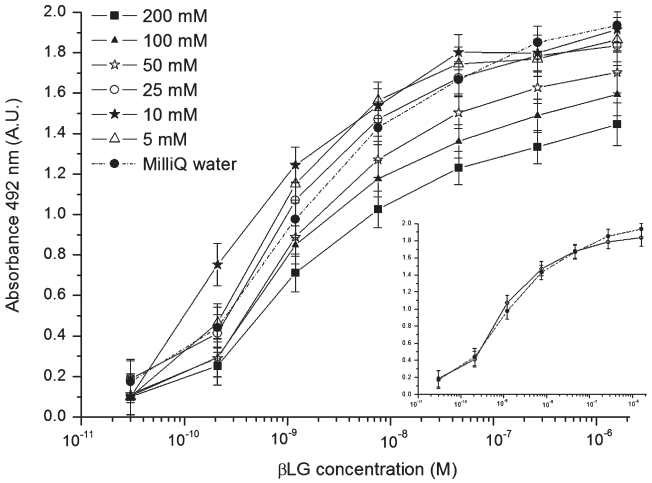


Figure 4. Curve obtained by ELISA under the optimized conditions for aqueous solutions of standard βLG and for standard βLG in PBS with different concentrations of GdnHCl in the solvent buffer, indicated by the different symbols in the figure. Assays were performed in triplicate. Vertical bars correspond to ± 2 SD values. (Inset) Aqueous solution of standard βLG and standard βLG in PBS with 25 mM GdnHCl.

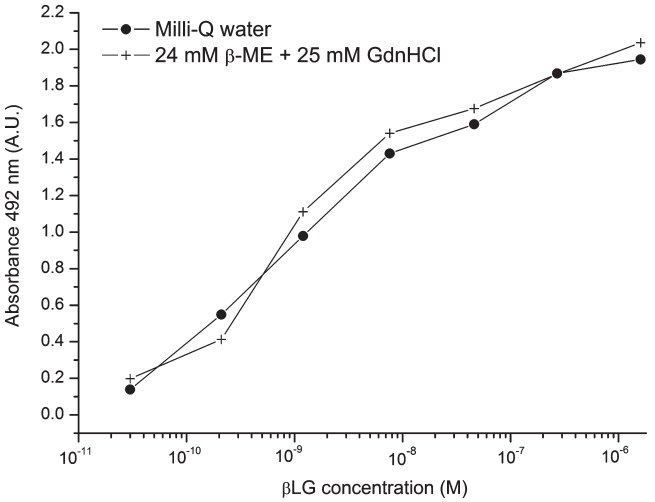


Figure 5. Curve obtained by ELISA under the optimized conditions for aqueous solutions of standard βLG and for standard βLG in PBS with βME and GdnHCl at selected concentrations.

βLG took place. The higher was the GdnHCl concentration, the lower was the absorbance of light (**Figure 4**). Thus, 25 mM was selected as the concentration for GdnHCl in the extraction solution because, as can be observed in **Figure 4**, this concentration of reagent does not affect the ELISA performance. It was also observed that the simultaneous addition of 24 mM βME and 25 mM GdnHCl to βLG did not affect the results of the ELISA (**Figure 5**). When GdnHCl was added to βME, borate buffer, and sodium chloride (procedure 6), results were significantly improved, as may be observed in **Table 2**. This result may be caused by GdnHCl, which makes specific regions of the βLG structure more accessible to the extraction reagents and also to the recognition sites of the antibodies in the ELISA procedure.

The best results in terms of the concentration of protein extracted and detected by ELISA were provided by the approach of diluting the sample in PBS solution instead of water (procedure 7) (**Table 2**) before the addition of the extraction agents (βME, borate buffer, GdnHCl, and sodium chloride). This difference in behavior is likely to be correlated with PBS facilitation of antigen−antibody interactions in the ELISA test. Furthermore, the presence of PBS, which increases the pH to 7.3, could facilitate βME disruption of βLG−casein bonding (*29*).

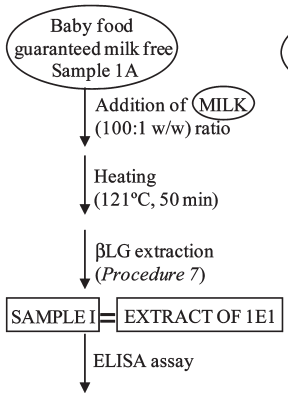
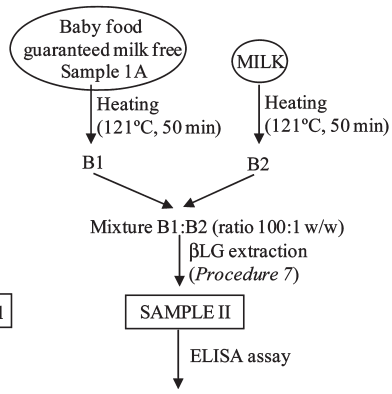
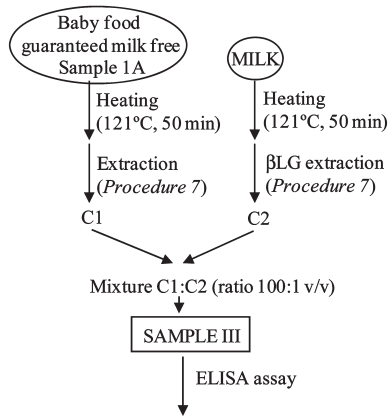
Each of the extraction procedures followed for sample 1E was tested in parallel with meat baby food guaranteed to be milk-free (sample 1A). The ELISA values for this sample were similar to those corresponding to blank assays, independent of the extraction procedure followed (see **Table 2**). Thus, no false positives were obtained by ELISA.

Influence of the Matrix and Thermal Treatment on the Recovery of βLG. The absence of certified samples of baby food contaminated with dairy products that contain a known amount of βLG precludes establishing the accuracy of the method of analysis. The accuracy calculated by spiking the sample 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 was contaminated with the βLG added (as dairy dessert) and heated afterward. As mentioned above, during the heat treatment of the processed foods βLG binds to casein micelles. In addition, other changes occurring as a result of industrial processing, such as glycosylation, affect aggregation of βLG (*32*). Depending on the specific nature of the food matrix, interactions with given compounds, such as retinol, β-ionone, and fatty acid lactones, can happen (*33*). For food of vegetal origin, phenolic compounds, such as flavonols and isoflavonols, can bind to βLG (*34*). It has been demonstrated that interactions between βLG and pectin from fruits can take place during the food processing (*35*), in which the formation and dissociation of complexes between βLG and pectin take place as a function of pH and salt concentration (*36*).

To study the influence of the matrix and thermal treatment on the recovery of βLG in samples containing dairy products, several extracted fractions described in **Table 3** were compared. These samples differ in the matrix that was in contact with the dairy product when heating or when extraction of βLG was performed. To prepare sample II, sample 1A and the aqueous suspension of powdered milk were individually heat-treated; after each had been manually homogenized, 100 g of baby food and 1 g of aqueous suspension of milk were mixed and submitted to procedure 7. For sample III, the same procedure (procedure 7) followed to extract βLG from samples containing it was applied to sample 1A, which did not contain dairy products, in order to extract any other component of the matrix, which would be extracted when using this procedure. The filtrates obtained by applying procedure 7 to the baby food and to the milk were mixed. The concentration of βLG detected in the samples by ELISA increased noticeably when the extraction of the baby food and of the dairy product took place independently before their mixing (βLG concentration detected in sample III > βLG concentration detected in sample II). Also, the concentration of βLG detected increased when the heating of the dairy product was performed in the absence, rather than in the presence, of the dairy product (concentration in sample II > concentration in sample I). These results seem to indicate that βLG extraction is hampered by the presence of the baby food matrix during the extraction process. On the other hand, heating the dairy product in the presence of the baby food matrix seems to favor βLG interaction with the components of the matrix, which makes the extraction of this protein more difficult.

Detection of βLG in Samples of Different Origins, at Different Contamination Levels, and in More Complex Matrices. To prove the feasibility of the sample preparation method developed to detect βLG in samples of different origins, in samples with lower levels of βLG contaminations, and in which the dairy product is a constituent of a dessert with other components, the extraction

Table 3. Influence of the Matrix and Heat Treatment on the Concentration of βLG Detected by ELISA in Samples Containing Dairy Products

DESCRIPTION OF THE PROCESS	Extraction of the chicken with rice baby food contaminated (100:1) with milk.	Extraction of mixture of the heated chicken with rice baby food βLG free and the heated milk.	Mixture of the extract of the heated chicken with rice baby food βLG free and the extract of the heated milk.
SCHEMATIC PROCESS			
DETECTED βLG (μg/g) Mean ± SD (n=4)	0.18 ± 0.01	1.34 ± 0.14	5.40 ± 0.56

method selected and the ELISA method optimized were applied to different samples. Those samples were prepared by deliberately contaminating different baby foods with both dairy products and dairy desserts (see **Table 1**). Adulterations were performed even at ratios as low as 1:100000 of dairy product to baby food. The comparison of the βLG concentration detected by ELISA employing the classical method (procedure 1) for sample preparation and the innovative one optimized in this work (procedure 7) for the samples studied is shown in **Table 1**. Although, as discussed above, it is not possible to determine the accuracy of the method, it can be seen that the protein extractability increases noticeably when the new sample preparation method is employed. Each datum is based on at least three independent extractions for each sample and two replicated measurements per extraction. To test the reproducibility of the method, repetitive analyses of the same samples prepared in the same conditions were performed. Results showed no significant difference between extraction RSD and measurement RSD with a confidence level of 95% ($\alpha = 0.05$).

The innovative method did not produce false positives. As already seen above for sample 3A, the absorbance obtained by ELISA for samples 1A, 2A, and 3A of the three matrices studied guaranteed to be βLG free were similar to that of the blank assay.

This new method allowed detecting βLG contamination in all of the samples up to a ratio as low as 1:1000 of dairy product to baby food. In some samples, levels as low as 1:100000 of dairy product to baby food (e.g., samples 1E4 and 2E4) have been detected, although they have not been quantified because the βLG content of these samples is inferior to the quantification limit of the ELISA. Conversely, the classical preparation method allowed detection of only the maximum level of contamination studied (1:100) for some types of samples, but not in all of them. In the case of samples 3E1 and 3D1 (fruit baby foods contaminated with yogurt or yogurt with pear, respectively, in a ratio of 1:100 yoghurt to baby food), in which false negatives were obtained with the classical sample preparation method, the presence of βLG is detected when using the innovative method.

For those samples for which βLG detection and quantification were possible with the innovative sample preparation procedure, contamination levels were differentiated. A significant example is the case of samples 2E1−2E3 (fish baby foods contaminated at different levels with fresh cheese). The βLG content for sample 2E1 was 3.00 μg/g (1.64×10^{-7} M). This result is about 1 order of magnitude higher than in sample 2E2 [0.24 μg/g (1.32×10^{-8} M)] and about 2 orders of magnitude higher than in sample 2E3 [0.047 μg/g (2.58×10^{-9} M)], which had been contaminated with an amount of dairy product 10 and 100 times lower, respectively (see **Table 1**).

Concluding Remarks. Optimization of the ELISA method in the sandwich format carried out in this study allows detecting standard βLG at the picomolar level. The sample preparation procedure developed for analyzing βLG in baby food makes it possible to detect by ELISA 1 part of dairy product (milk, cheese, or yogurt) in 1000 parts of baby food for all three types of these (meat, fish, or cereal and fruit) studied. Sensitivity is different for the diverse baby food samples analyzed, most probably due to the dependence of the extraction efficiency on the nature of the matrix and on the manufacturing conditions. Levels as low as 1 part of fresh cheese, as a constituent of the cheese dessert, in 100,000 parts of hake with rice baby food were detected. Fabrication of well-controlled deliberately contaminated baby foods, which resembles the potential contamination that could happen during manufacturing, has made it possible to perform a more realistic study than those carried out by spiking food with standard allergen.

The joint use of the developed sample preparation method and the optimized ELISA is a very promising tool for the food industry and regulatory agencies. It would allow them to verify the effectiveness of allergen avoidance procedures in manufacturing and ensure compliance with labeling guidelines. Moreover, the analytical approach proposed is easy to use and can be performed in any laboratory with basic equipment.

Additional studies to further improve the sensitivity of the method are being developed in our laboratory. The possibility of

coupling the sample preparation method to different analytical techniques is also being studied.

ABBREVIATIONS USED

βLG, β-lactoglobulin; βME, β-mercaptoethanol; ELISA, enzyme-linked immunosorbent assay; GdnHCl, guanidine hydrochloride; LOD, limit of detection; LOQ, lower limit of quantitation; OPD, *o*-phenylenediamine; HRP, horseradish peroxidase; PBS, phosphate buffer saline; OD, optical density.

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