“Capillary electrophoresis with laser induced fluorescence detection of proteins from two types of complex sample matrices: food and biological fluids”

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Summary

Sample preparation and laser induced fluorescence detection are two key steps of the analytical methodology to determine by capillary electrophoresis low concentrations of proteins in complex sample matrices. In this chapter the options of performing both steps in different ways are shown by detailing the analysis of the allergen β-lactoglobulin in food products for infants and the analysis of the isoforms of alpha 1-acid glycoprotein, a potential biomarker, in serum and secretome.

Keywords

Sample preparation, capillary electrophoresis, LIF, detection, lactoglobulin, alpha 1-acid glycoprotein, orosomucoid, milk, isoform, biomarker.

1. Introduction

At present, there is no doubt that capillary electrophoresis (CE) is a very powerful technique for protein analysis (***1***). This method features high resolution for separating proteins in complex samples as well as to separate closely related isoforms of a given protein related to its micro-heterogeneities.

However, when analyzing complex sample matrices, there are two important factors that should be considered in order to succeed in adequate analysis of proteins by CE. The first factor is the large number of compounds which are present in a broad concentration range, making almost impossible to detect a single analyte in spite of the high resolution CE can offer. The second factor to fight arises in those cases in which the proteins of interest are

present in a very low concentration. In this instance, the frequently used UV-Vis detection does not provide high enough sensitivity.

Two key steps to solve these problems to make possible carrying out the CE analysis of the proteins of interest are sample preparation and detection. Both of them can significantly contribute to increase the selectivity and the sensitivity required for a successful assay. Sample preparation is aimed at eliminating possibly interfering compounds as much as possible for the CE analysis. This step can also provide pre-concentration of the analytes of interest in the purified sample. The chosen detection mode should be sensitive enough to analyze the compounds of interest in the purified sample and, additionally, it can provide selectivity for some compounds. Protein analyses by CE are most commonly performed using UV detection due to the almost universal character of this approach and its affordable price. However, the limit of detection provided by UV detection for proteins can only reach the micromolar range, i.e., is not sensitive enough to analyze low concentration proteins of interest, such as trace amounts of allergens in food products or low copy level biomarkers in biological fluids. One of the most sensitive detection modes for CE is laser induced fluorescence (LIF) reaching detection limits as low as picomolar; however many proteins do not have native fluorescence thus they need to be fluorescently tagged with a fluorescent or fluorogenic dye prior to analysis (***2***). Important to note that labeling should be performed in such a way that it neither causes excessive band broadening nor multiple peaks for the species of interest as it would distort the CE separation.

The schematic representation of the procedures to perform sample preparation and consequent derivatization and CE-LIF analysis of different proteins in complex sample matrices is delineated in Figure 1.The first case corresponds to the analysis of the allergenic

protein β-lactoglobulin (βLG) in infant foods. The second case describes the analysis of the potential biomarker alpha 1-acid glycoprotein (AGP) isoforms in two biological fluids, namely serum and secretome.

[Figure 1 near here]

Food allergy is an increasing problem worldwide and the immunologic reaction to milk proteins is considered as one of the most common. Bovine βLG, a protein with a molecular mass of about 18 kDa and a pI of 5.09 for the variant A and pI of 5.23 for the variant B, is considered as the main allergenic protein in cow's milk (***3***), even when present at trace levels. Currently the only preventive measure for sensitized individuals is an avoidance diet. The recent regulations in the European Union (***4***) require that information about the presence of milk and its products in food even in altered forms, should be provided to the consumer. Besides of the presence of milk proteins as food ingredients, in some instances milk proteins are not purposely added, i.e., their presence is the result of contamination during storage or production. Thus, analysis methods selective and sensitive enough for detecting trace levels of the allergen βLG in different food products and even during production processes are greatly needed.

Our group has previously reported that sample preparation methods have noticeable effects on the sensitivity of ELISA and CE analyses for βLG (***5-7***). The composition of solvents used to extract βLG from the samples was designed taking into consideration the possibility of βLG being incorporated in casein micelles or to interact with other components of the food matrix when the food was submitted to heat treatment during processing. Thus, the recommended extraction solution contained a reducing agent (β–mercaptoethanol),

guanidine hydrochloride to make specific regions of the polypeptide chain more accessible, a buffer to regulate pH (borate), and high salt concentration (sodium chloride) to enhance βLG solubility. In the present chapter it is detailed how to apply this sample preparation method to determine βLG in three types of baby foods purposely contaminated with dairy products before submission to thermal treatment to simulate the cross-contamination that potentially could happen during production. Once the sample is prepared, on-capillary derivatization of proteins through their amino groups is performed and CE separation with LIF detection is carried out in the same capillary (on-line) (***7***) (see top part of Figure 1).

The bottom part of Figure 1 details the procedure for determining glycoprotein isoforms in biological fluids. Protein glycosylation depends, among other factors, on the pathophysiological conditions of the individual. Glycoprotein isoforms can serve as biomarkers in different major diseases such as cancer or vascular diseases. Alpha 1-acid glycoprotein (AGP) is a 41-43 kDa protein with pI in the range 2.8-3.8 with very high heterogeneity regarding both of its glycosilic and peptidic moieties (***8-10***). We have previously shown that in order to analyze AGP isoforms by CE, the target glycoprotein should be previously purified from the biological matrix (***11-12***). The high selectivity provided by the antibody-antigen recognition and the advantages of performing it on chromatographic format made immunoaffinity chromatography (IAC) the technique of choice for purifying AGP from serum or plasma (***13***). In the procedure detailed in the present chapter, AGP was purified by IAC from serum and secretome from an artery section of a patient suffering from a vascular disease. The purified glycoprotein was fluorescently derivatized via its thiol groups off-line (***14***) and the labeled protein was analyzed by CE-LIF.

2. Materials

2.1. Samples

1. Standards

β-lactoglobulin A+B (βLG), alpha 1-acid glycoprotein (AGP) from human plasma and rhodamine B (RhB) were from Sigma-Aldrich (Steinheim, Germany).

2. Samples

-Three types of baby foods (MEAT, FISH and FRUIT) were analyzed (Table 1). The main components on them were chicken with rice in MEAT type, hake with rice in the FISH type, and orange and banana with cereal in the FRUIT type. Samples named MEAT-0, FISH-0, and FRUIT-0 corresponded to baby foods of each type guaranteed free of dairy products. These matrices were on-purpose contaminated with dairy desserts in order to simulate contamination could occur during manufacturing. The contaminants were rice pudding (containing 65% of milk) for the sample MEAT-A, fresh cheese dessert with fruit (40% of fresh cheese) for the FISH-A sample, and yoghurt with pear (26% of yoghurt) for the FRUIT-A and FRUIT-B samples. After the contamination, the baby foods were subject to the same thermal treatment that they would experience if contamination had occurred during the production process (50 min at 121 °C for the MEAT type, 45 min at 123 °C for the FISH type, and 10 min at 105 °C for the FRUIT type samples). All samples were prepared for this study by Hero España, S.A. (Murcia, Spain).

[Table 1 near here]

- Serum and secretome. Human serum (provided by Hospital Ramon y Cajal, Madrid, Spain) and secretome from an artery section (provided by Fundacion Jimenez Diaz, Madrid, Spain) were used as samples. Obtaining of the samples was approved by the ethical committees of both hospitals. Serum was obtained by standard procedure (***15***). Secretome was obtained from an artery section of a patient suffering from abdominal aortic aneurysm by incubation of the tissue during 24 h in serum-free RPMI medium at 37°C and collecting the supernatant (this sample will be referred as secretome henceforth).

2.2. Sample preparation for βLG detection in infant foods

1. Concentrated phosphate buffer saline (PBSx10) (See Note 1).

2. β-mercaptoethanol, guanidine hydrochloride, and sodium tetraborate decahydrate (borax) were purchased from Sigma (St. Louis, MO, USA).

3. Sodium chloride was from Merck (Darmstadt, Germany).

4. Supernatant filtration: Whatman® 40 filter papers (Maidstone, UK) and syringe filters PVDF membrane 0.45 μm pore size from Millipore (Bedford, MA, USA).

5. Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.3. Sample preparation for analysis of AGP isoforms in serum and secretome

AGP from serum and secretome was purified by Immunoaffinity Chromatography (IAC) (***12-13, 15***).

1. Polyclonal goat anti-human AGP affinity purified was purchased from Immune Systems LTD (Devon, UK).

2. Epoxy-silica (Waters Protein-Pak Affinity epoxy-activated) of 40 μm particle size and 500 Å pore diameter was from Waters (Millipore, Waters Chromatography Division, Milford, MA, USA).

3. PEEK-lined stainless steel tubing (3 cm x 4.6 mm I.D.) (Grace Davison Discovery Science, Deerfield, IL, USA) provided with 1/4–1/16 reduction end fittings and with 2 μm pore diameter frits was used as IAC column.

4. Mobile phase and Binding buffer: Phosphate buffer saline (PBS) (See Note 1).

5. Desorption solution: Glycine-HCl pH 2.2. (See Note 2).

6. Neutralizing solution: 0.1 M Na_2HPO_4.

7. The HPLC system consisted of a Waters HPLC 510 pump (Milford, MA, USA), connected to two 7125 Rheodyne (Cotati, CA, USA) injection valves (named as V1 and V2) with polyetheretherketone (PEEK) injection loops of 1.4 mL and 0.2 mL volume, respectively. The IAC column was connected to the second injection valve and to the detector using PEEK tubing. A UV-Vis LC-95 detector from Perkin Elmer (Norwalk, CO, USA) set at 280 nm was employed. Detector signal was digitalized using a 406 Analog Interface module (Beckman Instruments, Fullerton, MO, USA) and a System Gold software version 8.1 (Beckman). A PEEK tube (length 4.5 cm, 1/16" O.D., 0.020" I.D.) connected to the detector outlet allowed manual collection of the sample fractions. This equipment was similar to the one shown in Fig. 1 of reference (***16***) except that in the present study the valve V3 and the detector at 450 nm were removed.

8. Centrifugal filter devices were used with nominal M_r 10,000 cut-off membrane and 0.5 mL volume either Microcon® 10 YM-10,(Millipore, Billerica, MA, USA) or Amicon®Ultra

0.5 mL 10K (Millipore, Carrigtwohill, Cork, Ireland) and with nominal M_r50,000 cut-off membrane and 2 mL volume Centricon®YM-50 (Amicon, Beverly, MA, USA) (See Note 3).

2.4. Fluorescent labeling for βLG detection in infant foods

1. Labeling reagent: 3-(2-Furoyl)quinoline-2-carboxaldehyde (FQ) was from Molecular Probes (Eugene, OR, USA) (See Note 4).

2. Nucleophilic agent for the derivatization with FQ: Potassium cyanide was from Sigma (See Note 5).

2.5. Fluorescent labeling for analysis of AGP isoforms in serum and secretome

1. Reducing solutions: 0.6 mM tris(2-carboxyethyl)phosphine (TCEP) (Sigma-Aldrich, Steinheim, Germany) in 133 mM ammonium bicarbonate and 0.06 mM TCEP in 200 mM ammonium bicarbonate (see Note 6).

2. Labeling solutions: 4.9 and 0.4 mM 5-(iodoacetamido)fluorescein (5-IAF, Sigma-Aldrich) in 100 mM ammonium bicarbonate and 20% acetonitrile to derivatize samples from serum and secretome, respectively (see Note 7).

3. Dithiothreitol (DTT) (Sigma-Aldrich) solution 1 M in water.

4. Ammonium bicarbonate (Fluka, Buchs, Germany): 100 mM solution in water, daily prepared.

5. Centrifugal filter devices Amicon® Ultra 0.5 mL 10K.

2.6. Capillary electrophoresis analysis for βLG detection in infant foods

1.The in house made CE apparatus with LIF detection employed previously described (***17***), was used with some modifications (***7***). Briefly, an RS/EH50R high voltage power supply

(Glassman High Voltage, Whitehouse Station, NJ, USA) was used in normal polarity configuration (anode connected at the inlet end of the capillary). Sample injection was carried out by raising the sample vial at a giving high during a giving time (injection by gravity). A 2060-10S Spectra Physics Ar-ion laser (9 mW) (Spectra Physics, Mountain View, CA, USA) was used as an excitation source at 488 nm in an orthogonal configuration. Fluorescence was collected using a 40x plan-achromatic microscope objective, passed 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, Mamamatsu City, Japan) operated at 600 V and assembled on top of a high-precision stage for alignment (Newport, Mountain View, CA, USA). Photocurrent was processed by a 7070 photometer (Oriel, Stratford, CT, USA) and a 406 System Gold A/D converter (Beckman, Fullerton, CA, USA). A laboratory-made 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). The heating device was made using two PVDF T-pieces model 1C300T03PV (EM-Technik, Maxdorf, Germany) interconnected by a 1 cm-long Tygon tube (6 mm O.D., 5 mm I.D.) using one entrance of each of the T-pieces. This assembly formed a jacket that allowed the insertion of the first 10 centimeters of the separation capillary through the distal entrances of the two T-pieces. This jacket allowed the control of the temperature in this length of the capillary using water from a circulating thermostatic bath entering the device through Tygon tube connected to the two remaining entrances of the T-pieces. Water-tightness of the device was achieved using PVDF cutting rings and thumb-nuts for Tygon tubing connections and silicone septa for the insertion of separation capillary.

2. 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 (See Note 8).

3. Separation buffer: 6 mM Borax, 6 mM SDS (pH 9.0).

4. Internal standard: Rhodamine B.

2.7. Capillary electrophoresis for analysis of AGP isoforms in serum and secretome

1. The instrument used for CZE separations was a G1600AX CE system from Agilent Technologies (Waghäusel, Germany). Sample injection was performed by applying pressure. LIF detection was performed by means of a ZetaLIF Discovery detector (Picometrics, Toulouse, France), connected to the CE instrument, and excitation was carried out by a PC 13589 model DPSS laser (Spectra Physics) at 488 nm with a nominal output power of 20 mW. Photomultiplier tube at the detector was set at 700 V (see Note 9).

2. Fused-silica capillaries 70 cm (51 cm to the detector) and 50 μm I.D. (375 μm O.D.) were supplied by Picometrics (see Note 10).

3. Background electrolyte (BGE): 10 mM Sodium acetate, 10 mM tricine, 10 mM sodium chloride, 7 M urea, and 4.5 mM putrescine pH adjusted at 4.5 (see Note 11).

3. Methods

3.1. Sample preparation for detection of βLG in baby foods

Suspend 1 g of the baby food sample in 10 mL of concentrated buffer saline solution (PBSx10) (See Note 1). Add an aliquot of 500 μL of the extracting solution containing a final concentration of 24 mM β-mercaptoethanol, 25 mM guanidine hydrochloride, 5% (v/v) of 2.5 mM borate buffer at pH 8.3, and 30 mL of 0.15 M sodium chloride. Shake the

mixture for 15 min at 80 ºC and centrifuge at 9000 x g for 25 min at room temperature. Collect the supernatant. Finally, filter it successively through a Whatman® 40 filter paper and through a syringe filter PVDF membrane 0.45 µm pore size.

3.2. Sample preparation for analysis of AGP isoforms in serum and secretome

The sample preparation method for AGP isoform analysis is similar to the one previously reported for plasma samples (***13***).

1. Immunoaffinity chromatography (IAC) anti-AGP column fabrication

- Binding of the antibody (Ab) to the packing material: Transform the epoxy groups of the supporting material into diol groups by adding 17.5 mL of 0.072 N sulfuric acid to 0.385 g Protein-Pack material and shaking for 1 h at room temperature. Discard the supernatant and wash the supporting material with water. Oxidize the formed diol groups to aldehyde by adding 5 mL of 0.23 M sodium periodate and keep for 2 h at room temperature. To stop the oxidation reaction, add 3.4 mL glycerol diluted with 15 mL of reaction solution (0.1 M sodium phosphate at pH 5.7). Discard the supernatant and wash the packing material with the reaction solution. Simultaneously, concentrate 3 mL of commercial antibody (1mg/mL) to 0.5 mL and transfer them to the reaction solution using a centrifugal filter device (Centricon®) with a 50 kDa nominal cut-off membrane (See Note 12). Add the concentrated 0.5 mL antibody plus the same volume of sodium cyanoborohydride to the supporting material. Shake the mixture for 65 h at 4 ºC. Discard the supernatant and wash the Ab-packing material with 0.1 M sodium phosphate (pH 7). Reduce the unreacted aldehyde groups with 7 mg of sodium borohydride. Wash the Ab-derivatized Protein-Pack material with 0.1 M sodium phosphate at pH 7, then with a solution of pH 7 containing 0.1 M sodium phosphate and 0.5 M sodium chloride, and finally with a solution of PBS with

0.02 % (w/v) sodium azide. Keep the Ab-packing material at 4 ºC until packing into the HPLC tubing.

- Packing the Ab-support.

Prepare a packing device consisting of a precolumn (0.63 mL volume) of the same internal diameter that of the column connected with a zero dead volume union to a larger diameter tube used as packing reservoir (13.5 mL volume). Remove the top 1/4–1/16 reduction end fitting and the frit from the column tube and connect this column tube to the precolumn with a zero dead volume union. Keep the system reservoir-precolumn-column vertical and fill the column with PBS. Mix the derivatized packing material with about 8 mL PBS and stir manually. Pour the slurry into the reservoir avoiding support settling. Fill quickly the reservoir with PBS, connect it into the HPLC pump and flush PBS through the deposit-precolumn-column system at increasing flow rate until reaching 10 mL/min. Keep this flow rate for 15 min. Afterwards, slowly decrease the flow rate until reaching 0 mL/min, stop the pump and keep it in this way for 30 min. Remove carefully the precolumn and the reservoir. Place the frit and the 1/4–1/16 reduction end fitting at the column inlet. Flush the column with PBS plus 0.02% (w/v) sodium azide and keep it at 4 ºC until use.

2. AGP purification from serum and secretome.

-Sample pre-treatment: Incubate 0.1 mL of sample (either serum or secretome) with 1% (v:v) Protease Inhibitor Cocktail (Sigma) for 30 min at -20 °C and then dilute the incubated sample to 0.2 mL with Milli-Q water.

-Immunopurification process: At the beginning of the working day, right after the column is installed in the chromatographic system, condition the IAC column by pumping through PBS at 0.5 mL/min for 5 min and clean it with 3 injections of 1.4 mL each of Gly-HCl

desorption solution. When the baseline is stabilized (t = 0), inject the 0.2 mL pre-treated sample using the small loop in valve V2. After 10 min (t = 10 min), when the absorbance signal returned to baseline level (because the unbound components of the sample have been eluted), inject 1.4 mL of desorption solution using the large loop in valve V1. Collect the desorbed fraction, which contains AGP, and neutralize it with 0.1 M Na_2HPO_4 (see Note 13). At t = 30 min, reinject this neutralized fraction in the IAC column using the large loop in valve V1. At t = 40 min inject 1.4 mL of desorption solution. Collect the desorbed fraction, which contains purified AGP, and neutralize it with 0.1 M Na_2HPO_4. Inject 2 x 1.4 mL desorption agent and wait until the signal returns to baseline level to perform a new immunopurification process of another sample. At the beginning of each working day, clean the column by injecting 3 x 1.4 mL desorption agent. The column is stored at 4 ºC in PBS plus 0.02% (w/v) sodium azide overnight.

3. Salt removal and concentration are performed by using centrifuge filer devices with 10 kDa cutt off membranes (See Note 14).

3.3. On-line fluorescent protein labeling and capillary electrophoresis analysis with laser induced fluorescence detection of βLG from baby foods

The procedure for on-capillary derivatization is based on previously optimized method (***18***).

1. 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 from Hamilton (Bonaduz, Switzerland).

2. To properly assign the βLG peak, add rhodamine B to the sample prepared as indicated in section 3.1, so that the final concentration of this internal standard (I.S.) in the sample is 10^{-7} M.

3. Inject a plug of a mixture of the sample spiked with rhodamine B plus a KCN solution at final concentration 10 mM into the capillary. Inject 5 mM solution of FQ in separation buffer. Both injections are performed by gravity (20 cm height) for12 s (See Note 15).

4. Mixing step: Place the vials containing the separation buffer in the inlet and outlet ends of the capillary and apply high voltage of 3 kV for 6 min. Perform the step at 65 ºC by using the device described in section 2.6.

5. Reaction step: Switch off the power supply for 15 s. Perform the step at 65 ºC.

6. Separation step: Apply 15 kV at room temperature (See Note 16).

7. Collect the electropherogram showing in the X axis the migration time of the peaks relative to the migration time of rhodamine B.

8. Compare the relative migration time of each peak to that of a sample of standard βLG analyzed in the same way. Assign the peak of βLG in the samples to the one with the same relative migration time than standard βLG. In this way the results shown in Figure 2 and Table 2 for identifying the βLG peak in each sample are obtained. In the figure peaks a and b migrating close to the βLG peak and arising from other components of the sample are observed; the values of migration time relative to the internal standard calculated in Table 2 permit assigning the βLG peak.

[Figure 2 and Table 2 near here]

3.4. Off-line fluorescent protein labeling for AGP isoform analysis from serum and secretome

1. Warm up the samples prepared as described in section 3.2 to room temperature. Gently vortex the microtubes containing the samples for homogenization.

2. Add the corresponding reducing solution to each sample: 15 µL of 0.6 mM TCEP in the case of 200 µM AGP from serum and 5 µL of 0.06 mM TCEP in the case of 7 µM AGP from secretome (see Note 6).

3. Keep the samples at 37°C for 1 hour in a thermostatic bath to reduce disulfide bridges.

4. Add the corresponding labeling solution to each sample: 20 µL of 4.9 mM 5-IAF in the case of AGP from serum and 10 µL of 0.4 mM 5-IAF in the case of AGP from secretome (see Notes 7 and 17).

5. Keep the mixture at 37°C for 2.5 hours in a thermostatic bath.

6. After the labeling reaction, remove the sample from the bath and add 5 µL of 1 M DTT solution.

7. Keep the mixture at room temperature for 30 minutes to quench the labeling reaction.

8. Apply the mixture into an Amicon® Ultra 10K centrifugal filter device and add 100 µL of 100 mM ammonium bicarbonate solution. Centrifuge at 14000 x g for 30 minutes at 4°C (see Note 18).

9. Discard the filtrate and add 100 µL of 100 mM ammonium bicarbonate solution. Repeat centrifugation.

10. Discard the filtrate and wash the labeled glycoprotein by the addition of 100 µL of water to the centrifugal filter device each time, until the filtrate is colorless. Centrifugation conditions as in step 8 (see Notes 18 and 19).

11. Discard the filtrate and place the device containing the labeled glycoprotein into a new microtube at recovering position (upside down). Centrifuge at 1000 x g for 3 minutes at 4°C.

12. Recover the labeled AGP in water (15-20 µL), place it into a deactivated glass sample injection vial, and store at -20°C until the CZE-LIF analysis.

3.5. Capillary electrophoresis with laser induced fluorescence detection to analyze the AGP isoforms from serum and secretome

CZE-LIF analysis of AGP isoforms is carried out according to a previously published CZE-UV method (***19***), with some modifications.

1. Condition new capillaries as follows: rinse by pressure at 1 bar with 1 M NaOH for 30 min, water for 5 min, and finally 0.1 M NaOH for 15 min.

2. Between sample runs, rinse the capillaries by pressure at 1 bar with water for 5 min, 0.1 M NaOH for 10 min, water for 5 min, and finally by the BGE for 5 min.

3. Introduce the labeled sample into the capillary by hydrodynamic injection at 35 mbar for 30 seconds (injected volume is approximately 32 nL).

4. Perform CZE separation at 25 kV (357 V/cm) in normal polarity (anode at injection) at 35°C. An example of the expected results is shown in Figure 3, exhibiting the different isoforms of AGP from serum and secretome samples analyzed by CE-LIF, which are very similar to the ones observed by CE-UV for non-labeled standard AGP.

[Figure 3 near here]

4. Notes

Note 1.PBS consists of 0.01 M disodium hydrogenphosphate/sodium dihydrogenphosphate, 0.138 M NaCl, 2.7 mM KCl, pH 7.4. Ten times concentrated PBS (PBS x 10) was prepared and from it PBS was prepared daily by diluting 1 part of (PBSx10) with 9 parts of Milli-Q water. PBSx10 was prepared as follows: A solution A consisting of 0.1 M Na_2HPO_4, 1.38 M NaCl and 27 mM KCl, and a solution B consisting of 0.1 M NaH_2PO_4, 1.38 M NaCl and 27 mM KCl, were prepared. Solutions A and B were mixed until reached pH 6.85. Be aware, that pH can be modified by the dilution process. For this reason (PBSx10) was made at pH 6.85 so after 10x dilution the pH was 7.4.

Note 2.Gly-HCl pH 2.2 is made as follows: Prepare 500 mL of 0.1 M glycine containing 885 µL of pure HCl (Hydrochloric acid 35 % purissimum). After that, add drop by drop 2 N HCl until pH 2.2 is reached.

Note 3.The minimum volume in which the sample can be recovered by Microcon® 10 YM-10 and by Amicon® Ultra 0.5 mL 10K, is 5 µL and 15-20 µL, respectively, due to the design of the filter device. Thus, Microcon® 10 YM-10 devices are suggested to desalt and concentrate samples with low concentration of target analyte as it is the case of secretome samples.

Note 4. Preparation of FQ solutions: A 50 mM stock solution of FQ was prepared in methanol (HPLC grade, Scharlau Chemie, Barcelona, Spain). Since FQ degrades in solution even when stored at -20 ºC 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

aliquots were stored at -20 ºC until use. On the day of the experiment, the aliquots were defrosted and solved in the running buffer to obtain a 5 mM FQ concentration.

Note 5. Potassium cyanide is highly poisonous and reacts directly with acids to form lethal HCN gas. Therefore, stock solutions should be made in a basic buffer. Neutralization of waste containing KCN solution should be done by the addition of 1% NaOH solution (about 50 μL NaOH/g cyanide) followed by slow addition of bleach (about 70 μL bleach/g of cyanide).

Note 6. Diluted solutions of TCEP in water are unstable; therefore, reducing solutions are freshly prepared for each labeling reaction. The appropriate amount of TCEP is dissolved in the respective solution of ammonium bicarbonate. Reducing agent is added to keep a ratio 2:1 in respect to the number of cysteine residues in the glycoprotein, because if large excess is added, TCEP can react with the fluorescent dye. Solution pH is unadjusted. The amount of ammonium bicarbonate is calculated to obtain a final concentration of 100 mM bicarbonate in the mixture TCEP in bicarbonate plus AGP. For the samples in this study: 5 μL of AGP purified from serum (approximate AGP concentration 200 μM) were mixed with 15 μL of 0.6 mM TCEP in 133 mM ammonium bicarbonate. For AGP purified from secretome, 5 μL (approximate AGP concentration 7 μM) were mixed with 0.06 mM TCEP in 5 μL of 200 mM ammonium bicarbonate.

Note 7. To prepare 4.9 mM 5-IAF labeling solution, 0.5 mg of the dye is dissolved in 200 μL medium containing 100 mM ammonium bicarbonate and 20% acetonitrile, pH unadjusted, to ensure complete solubilization of 5-IAF. The labeling solution is prepared in a microtube by vortexing. The 0.4 mM 5-IAF solution is prepared by dilution in the same

medium. All the labeling solutions are prepared daily and stored in darkness until use. The 4.9 mM solution is used to derivatize the 200 μM AGP purified from serum. The 0.4 mM solution is used to label the 7 μM AGP purified from secretome.

Note 8. Capillaries are purchased in reels and cut to the desired length. Detection window is made by burning.

Note 9. The fluorescence signal is collected and passed through a long-pass filter (> 498 nm, as specified by Picometrics) in order to filter out any excitation light.

Note 10. Capillaries used in the Picometrics device are equipped with an ellipsoid mirror glued to the capillary window in order to collect the maximum amount of fluorescence signal from the sample.

Note 11. BGE is weekly prepared and stored at 4°C. As putrescine is unstable at low concentrations, a 1 M stock solution is made and stored at 4°C, of which 225 μL are employed to prepare the BGE. The pH of the solution is adjusted to 4.5 by the addition of 2 M acetic acid, and finally, the volume is completed to 50 mL with water.

Note 12. Buffer exchange and concentration of the antibody by centrifuge filter devices (Centricon®) with a 50 kDa nominal cut-off membrane (YM-50): Before using the YM-50 devices, they are passivated with a solution of 5 % (w/v) Brij® 35 for12 h to avoid any non-specific adsorption of the antibody to the device (***20***). After that, rinse the filter device with Milli-Q water (3 x 2 mL) and wash the membrane by 2 mL Milli-Q water at 4000 x g, 4°C, for 15 min . Set the filter device in recovery mode (upside down) and centrifuge it at 573 x g at 4°C for 2 min to remove the remaining water from the membrane. Set the filter

device in concentrating position and add the antibody solution. As the maximum volume of the device is 2 mL and the antibody volume is 3 mL, this concentration step is carried out by adding first 2 mL and concentrate, then add 1 mL. Concentration is performed by centrifuging at 4000 x g at 4°C for 50 min each time. After that, add reaction buffer (2 x 0.2 mL) and spin at 4000 x g at 4°C for 15 min. Set the filter device in recovery mode and spin at 800 x g at 4°C, for 2 min, to recover the concentrated antibody. Add 0.1 mL of reaction buffer onto the membrane of the filter device and recover the remaining antibody from it by turning the device upside down (800 x g at 4°C for 2 min). Repeat the last step. Mix the 3 fractions containing the recovered concentrated antibody and add reaction buffer to get 0.5 mL final volume.

Note 13. Neutralization of the fraction collected from the IAC column: The desorbed fraction is collected for 1.5 min at 0.5 mL/min flow rate and neutralized by adding 0.4 - 0.5 mL of 0.1 M Na_2HPO_4. Neutralization is checked by pH paper.

Note 14. Salt removal and concentration: Place the immunopurified and neutralized AGP sample (volume about 1.2 mL) in a centrifugal filter device with a 10 kDa cut off membrane (either Amicon® Ultra 0.5 mL 10K or Microcon® 10, though Microcon® 10 should be used for AGP from secretome, see note 3). Because the maximum volume for the device is 0.5 mL, the process is performed by spinning 0.5 mL volumes, then adding another fraction of the sample to the same vial and spin again, and finally, adding the rest of the sample and spin it. Every time a fraction is placed in the device centrifuge it at 13900 x g for 25-30 min at 4 ºC. After concentrating the whole sample, rinse the AGP retained in the membrane of the filter device by centrifuging 3 x 0.5 mL of Milli-Q water at 13900 x g

for 30 min at 4°C. When there is almost no liquid on the membrane, set the device in the recovery mode (upside down) and centrifuge at 1000 x g for 3 min at 4°C to recover the sample in the minimum volume that is usually about 20 - 30 µL for the Amicon® Ultra devices and 5 µL for the Microcon® devices.

Note 15. Wash the inlet end of the capillary by immersion into a vial containing Milli-Q water, after each injection of either sample plus KCN or FQ.

Note 16. Room temperature was set at 24 ºC.

Note 17. Labeling dye 5-IAF is added at a ratio 30:1 in respect to moles of cysteine residues in AGP, assuming a complete reducing process. It is not necessary to extract the excess of TCEP from the reaction mixture, despite it could react with the dye, because the reducing agent is not added at a large excess (see note 6). This procedure considerably simplifies the labeling process.

Note 18. Centrifugal filter devices allow the extraction of low molecular weight compounds from the labeling mixture as TCEP and 5-IAF, which could interfere with the subsequent CZE analysis. The addition of ammonium bicarbonate solution makes the elimination of the dye easier (higher solubilization at alkaline pH). Centrifugation time can be slightly shorter or longer, but in general, it is preferable to reduce the volume of the sample as much as possible, typically to 15-20 µL with the devices employed.

Note 19. Normally, three cycles of washing with water are enough.

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Acknowledgements

Isabel Vasallo from Hero Institute for Infant Nutrition (Hero España, S.A., Murcia, Spain) is acknowledged for preparing the infant food samples. Financial support from the Spanish Ministry of Science and Innovation (project CTQ2009-09399), CSIC (project 2010JP0013), and the Hungarian Government (project OTKA K-81839) is acknowledged. Angel Puerta acknowledges CSIC for a contract in the JAE doc program cofinanced by the European Union under the European Social Fund, and Raul Garrido-Medina acknowledges the Spanish Ministry of Education the Ph.D. fellowship (FPU) awarded.

Figure captions

Figure 1. Schematic representation of the two ways to perform sample preparation, fluorescent labeling and capillary electrophoresis with laser induced fluorescence detection for β-lactoglobulin (βLG) in baby foods and alpha 1-acid glycoprotein (AGP) isoforms in serum and secretome samples.

Figure 2. CE-LIF analysis of the extracts of contaminated baby food samples (FISH-A and MEAT-A) and non-contaminated (FISH-0 and MEAT-0) with dairy products. Peaks a and b are indicated in Table 2. I.S. corresponding to RhB. Adapted from Analytica Chimica Acta, 649, C. Pelaez-Lorenzo, J.C. Diez-Masa, I. Vasallo, M. de Frutos, 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, 202-210, Copyright (2009), with permission from Elsevier.

Figure 3. CZE analysis of the isoforms of (A) standard AGP (0.4 mM) with UV detection, (B) and (C) 5-IAF labeled AGP purified from (B) serum and (C) secretome, both with LIF detection. BGE: 10 mM sodium acetate, 10 mM tricine, 10 mM sodium chloride, 7 M urea, and 4.5 mM putrescine, pH adjusted to 4.5. Conditions for CZE-UV: hydrodynamic injection at 35 mbar for 25 seconds, 59.5 cm total length, 51 cm effective length, V = 21.2 kV, 35°C, detection wavelength 214 nm. Conditions for CZE-LIF: hydrodynamic injection at 35 mbar for 30 seconds, 70 cm total length, 51 cm effective length, V=25 kV, 35°C, and LIF detection. Adapted from R. Garrido-Medina, A. Puerta, Z. Rivera-Monroy, M. de Frutos, A. Guttman, J.C. Diez-Masa: Analysis of alpha-1-acid glycoprotein isoforms using CE-LIF with fluorescent thiol-derivatization. Electrophoresis. 2012.

Figure 1.

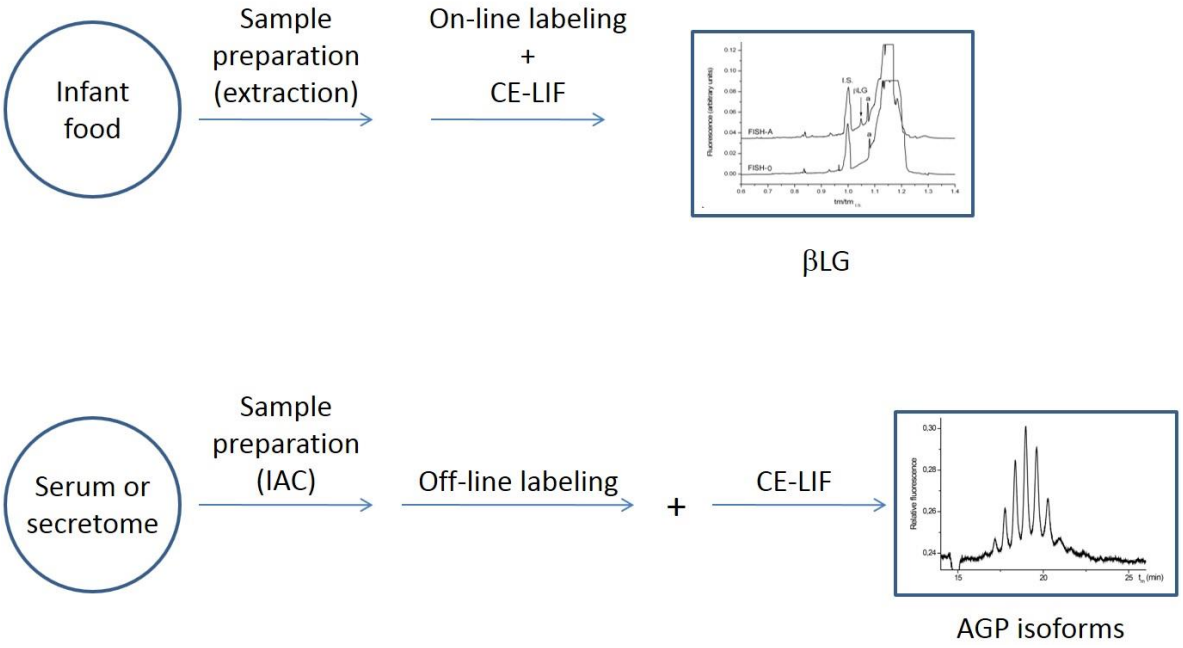
Infant food
Sample preparation (extraction)
On-line labeling + CE-LIF
I.S.
βLG
FISH-A
FISH-0
βLG
Serum or secretome
Sample preparation (IAC)
Off-line labeling
+
CE-LIF
Relative fluorescence
AGP isoforms

Figure 2.

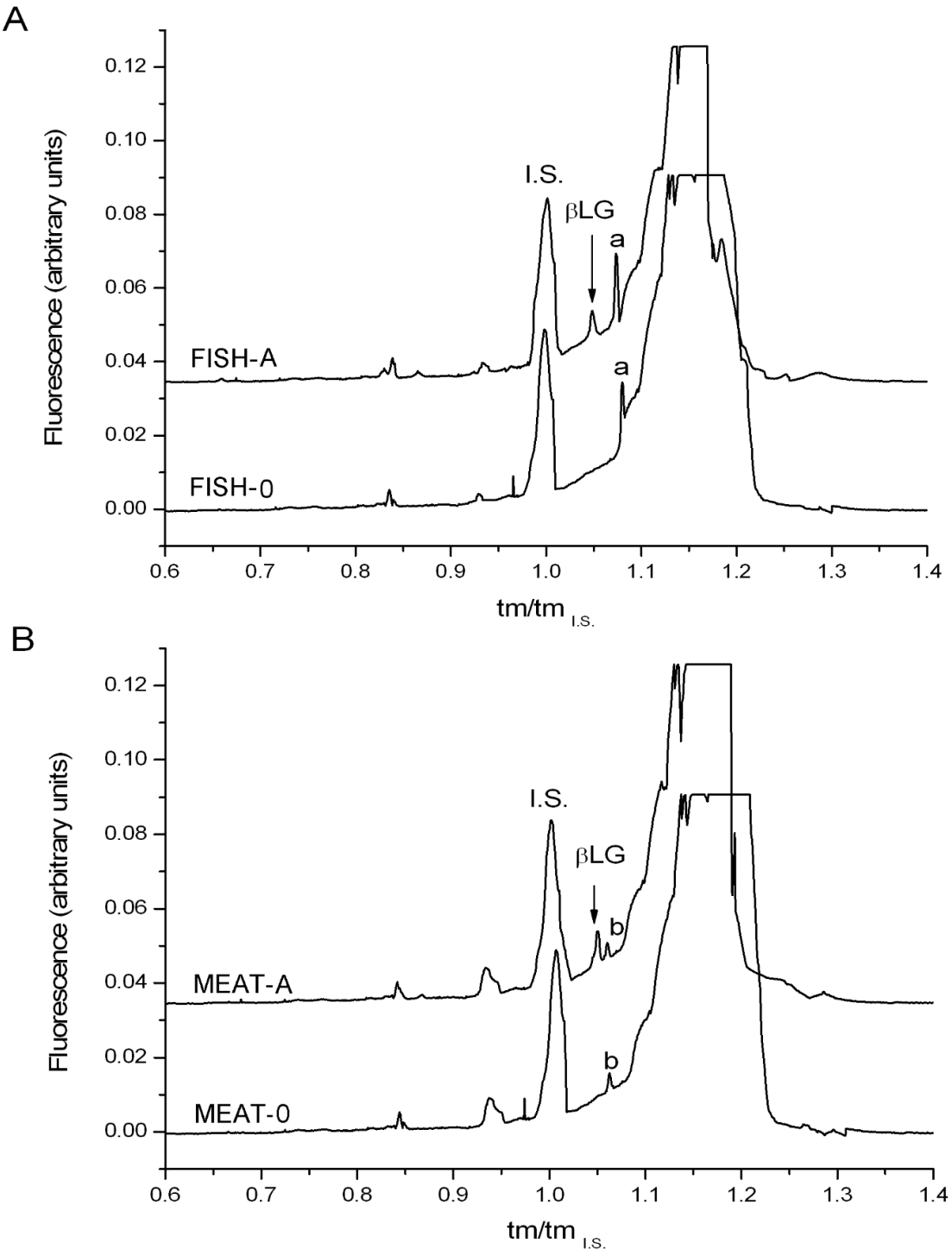
A
Fluorescence (arbitrary units)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
I.S.
βLG
a
a
FISH-A
FISH-0
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
tm/tm I.S.
B
Fluorescence (arbitrary units)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
I.S.
βLG
b
b
MEAT-A
MEAT-0
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
tm/tm I.S.

Figure 3.

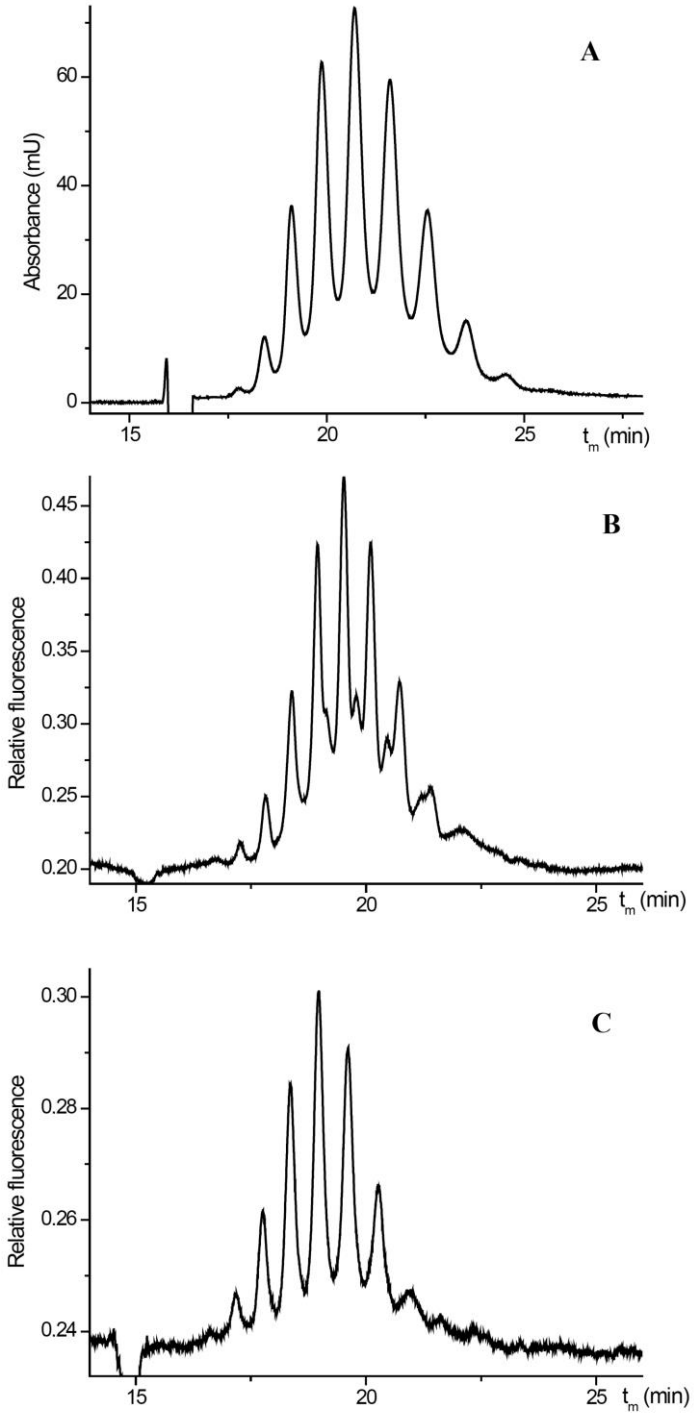
A
Absorbance (mU)
60
40
20
0
15
20
25
t_m (min)
B
Relative fluorescence
0.45
0.40
0.35
0.30
0.25
0.20
15
20
25
t_m (min)
C
Relative fluorescence
0.30
0.28
0.26
0.24
15
20
25
t_m (min)

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	None	Guaranteed free of dairy product	0	FRUIT-0
Orange and banana with cereal	Yoghurt with pear	1:260	1:1 000	FRUIT-A
Orange and banana with cereal	Yoghurt with pear	1:2 600	1:10 000	FRUIT-B
Hake with rice	None	Guaranteed free of dairy product	0	FISH-0
Hake with rice	Fresh cheese dessert with fruit	1:400	1:1 000	FISH-A
Chicken with rice	None	Guaranteed free of dairy product	0	MEAT-0
Chicken with rice	Rice pudding	1:650	1:1 000	MEAT-A

[a] Ratios calculated knowing the percentage of each dairy product in the dairy dessert: 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.

Adapted from Analytica Chimica Acta, 649, C. Pelaez-Lorenzo, J.C. Diez-Masa, I. Vasallo, M. de Frutos, 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, 202-210, Copyright (2009), with permission from Elsevier.

Table 2. CE-LIF migration time (tm) and migration time relative to the I.S. ($tm/tm_{I.S.}$) for the peaks of interest in Fig. 2.

Sample	**$tm_{I.S.}$** (min)	**$tm_{\beta LG}$** (min)	**$tm_{\beta LG}$/ $tm_{I.S.}$**	**$tm_{peak\ a}$** (min)	**$tm_{peak\ a}$/ $tm_{I.S.}$**	**$tm_{peak\ b}$** (min)	**$tm_{peak\ b}$/ $tm_{I.S.}$**
FRUIT-A	8.793	9.194	1.046	n.o.	n.o.	n.o.	n.o.
FRUIT-B	8.774	9.197	1.048	n.o.	n.o.	n.o.	n.o.
FRUIT-0	9.694	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.
FISH-A	8.562	8.957	1.046	9.240	1.079	n.o.	n.o.
FISH-0	8.517	n.o.	n.o.	9.195	1.080	n.o.	n.o.
MEAT-A	8.643	9.050	1.047	n.o.	n.o.	9.134	1.057
MEAT-0	8.561	n.o.	n.o.	n.o.	n.o.	9.053	1.057

$tm_{I.S.}$: 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.

n.o.: Not observed.

Adapted from Analytica Chimica Acta, 649, C. Pelaez-Lorenzo, J.C. Diez-Masa, I. Vasallo, M. de Frutos, 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, 202-210, Copyright (2009), with permission from Elsevier.