Development of a novel duplex lateral flow test for simultaneous detection of casein and β-lactoglobulin in food

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Abstract

Milk by-products such as whey and caseinate are widely used as ingredients or processing aids in food industry. However, since they could cause allergic reactions they are included in Allergen Control Plans. β-lactoglobulin is the major whey protein and caseins are main proteins in milk. Selection of a unique target to analyze the presence of milk in foods could be insufficient when the source of milk proteins is unknown. A new test based on lateral flow immunocromatography that combines the simultaneous and independent detection of both proteins (β-lactoglobulin and casein) in one rapid test was developed. The assay was validated according to AOAC guidelines being able to detect β-lactoglobulin (0.5 ppm), casein (2 ppm), whey and powder milk (1-5 ppm). No cross-reactivity was found with a panel of 38 food commodities. The method is a rapid and suitable tool to identify milk proteins in processed food, ingredients, and rinsing water.

Keywords

Milk allergens, multiplex, immunochromatography, HACCP

1. Introduction

Milk is widely used as an ingredient in food industry due to its nutritive value and technological properties. Moreover, milk is a well-balanced nutrient that is introduced in many meals for children. In order to protect consumers from pathogenic bacteria, milk is treated using different thermal processes: pasteurisation, in-container-bottle sterilisation or ultra-high temperature (UHT) treatment (Claeys et al., 2013; EFSA Panel on Dietetic Products, Nutrition and Allergies, 2014). Moreover, milk can also be processed by evaporation, spray drying, filtration, hydrolysis and fermentation during the manufacturing of different dairy products, such as cheese, yogurt, whey concentrate powder or caseinate (Verhoeckx et al., 2015). Some of them are ready to eat and others are added as ingredients or used as processing aids by food manufacturers. An example of the last one is the use of caseinates in the manufacturing of alcoholic beverages (beer, wine) as clarification processing aids. Wines fined with this product, even after filtration, may trigger adverse reactions in susceptible individuals (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2014).

In children under 3 years milk was identified as one of the main allergenic sources (Fernández-Rivas, 2009). Milk allergy prevalence in children under 3 years ranged 2-7.5% in Europe, 2.2% in Canada, 6.5% in Australia and 13.4% in EEUU (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2014). However, it is reduced when children grow up as a result of development of clinical tolerance to milk proteins. Symptoms of this allergy can vary from mild, just a skin rash, to severe reactions with the most serious manifestation of anaphylactic shock (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2014). Although food labelling and control related to food allergens is different across the countries, milk is included among the main 8 foods that should be considered for allergen labelling by the Codex Alimentarius commission (FAO, 2016) and in the legislation of most countries such as the European Union, USA, Canada, Japan or

47 Australia (Gendel, 2012). In the past years, food industries have done huge efforts to include
48 allergens in their Hazard Analysis Critical Control Point (HACCP) plans (Gupta et al., 2017).
49 However, despite such effort, around 10% of reported food alerts in Europe are still due to the
50 presence of undeclared allergens on food labels (RASFF, 2017). In USA, undeclared food
51 allergens reached 47% of all commercial foodstuffs in 2013-2014 (Do, Khuda, & Sharma, 2018).
52 As long as milk is frequently used as an ingredient or a processing aid in food industry, a great
53 risk of unintentional presence of this allergen exists. Therefore, the development of rapid and
54 easy-to-use tools can help food industry to control the risk of cross-contaminations as well as
55 to verify the presence of this ingredient in raw materials and final products.

56 Among the milk proteins, caseins and whey proteins such as β-lactoglobulin or α-lactalbumin
57 are the most allergenic. The choice of the most suitable target to detect the presence of milk
58 proteins depends on several factors, the abundance, the thermal stability and the frequency of
59 use as ingredient in the manufacturing of foods, among others. Caseins account for 80% of
60 total proteins in milk; thus respect to abundance, these proteins could be considered as the
61 best target for milk detection. Meanwhile, β-lactoglobulin is the major whey protein. Caseins
62 are poorly affected by thermal processes while whey proteins can be denatured, which could
63 affect their allergenicity (Verhoeckx et al., 2015) and immunodetection (de Luis, Lavilla,
64 Sánchez, Calvo, & Pérez, 2009). However, casein presence is low in some milk by-products,
65 such as whey protein isolates or concentrates, which are a very common ingredient in the
66 manufacturing of many foods. Therefore, detection of milk in food is a challenge and analysing
67 just one of the two types of proteins as the target could be insufficient to assure the absence
68 of milk, especially when the source of milk proteins is unknown.

69 Although many methods for detecting food allergens have been developed, only some of them
70 have a practical application in food industry or a use restricted to some specific conditions.

According to FoodDrinkEurope (2013) and Walker (2019), ELISA technique should be used for mapping the sources of allergen contamination in a facility, to validate the cleaning processes or to control raw materials and final products since quantitative results may be obtained. However, for routine cleaning verification checks and for testing finished products on site Lateral flow immunoassays (LFIA) could be used due to easy to use, quick response and affordable cost. PCR methods should only be used where no other protein detection technology is available. Finally, although mass spectrometric methodology is being optimized for routine allergen food analysis (Monaci, De Angelis, Montemurro, & Pilolli, 2018), it could be used when secondary confirmatory techniques are required (Walker, 2019).

LFIA technique allows a suitable detection of allergenic proteins without the need of trained operators and well-equipped laboratories, making it a convenient tool to be included in the HACCP plans (Dzantiev, Byzova, Urusov, & Zherdev, 2014). In addition, due to its quick response (5-10 min), this method allows *in situ* testing and hence taking corrective actions in a short time. Up to date, several LFIA tests have been developed to detect casein and/or β-lactoglobulin and their weaknesses and strengths were recently evaluated (Courtney, Taylor, & Baumert, 2016). In some cases, test manufacturers claim for detecting total milk proteins in their products. However, most of them were not able to detect whey proteins but just only casein. A similar issue was found by using commercial ELISA tests when whey protein concentrates (WPC) were tested with total milk protein kits (Ivens, Baumert, & Taylor, 2016). Thus, a great concern is generated when a kit based on the detection of total milk is used to detect whey ingredients (Courtney et al., 2016; Ivens et al., 2016). The availability of tests which allow the simultaneous detection of both, casein and whey proteins, could help food operators to get a complete testing of milk residues in food commodities, independently of the type of milk proteins added or present by cross-contamination. In addition, the identification of caseins or β-lactoglobulin in independent lines provides very useful information about the

source of contamination, but so far two different tests have to be used to identify the presence of both milk protein components. In the last years, the development of duplex (or even multiplex) assays based on lateral flow immunocromatography has increased due to the advantage of simultaneous detection of several analytes in the same assay. Thus, this format is excellent for saving time and cost, and for improving control efficiency. Some recent studies have been focused on mycotoxin detection using multiplex LFIA (Song et al., 2014; Zhang et al., 2017). However, up to date no multiplex test has been developed to detect allergens in foods (Ross, Bremer, & Nielen, 2018).

The aim of this work has been to develop and evaluate the performance of a novel duplex lateral flow test for the simultaneous and independent detection of casein and β-lactoglobulin in a unique LFIA strip. Validation was performed following the AOAC guidelines for qualitative binary chemistry methods. The POD (Probability Of Detection) was determined at different levels of the specific target proteins as well as in UHT milk. Cross-reactivity, robustness and lot-to-lot variation of the test were also evaluated in a single laboratory validation.

2. Materials and methods

2.1 Antibody preparation

Affinity purified polyclonal antibodies against β-lactoglobulin were obtained as previously described (de Luis et al., 2008). Antibodies against β-casein and Internalin A were raised in rabbits using an approved protocol by the Ethic Committee for Animal Experiments from the University of Zaragoza (Project Licence PI65/14). Antibodies were purified by affinity chromatography against the correspondent protein insolubilized in Shepharose (HiTrapNHS-

activated HP column, GE) using an AKTAprime plus equipment (GE) and stored at -20 °C until used.

2.2 *Preparation of* dyed latex *particle conjugates for LFIA*

Red and Blue Carboxyl-Modified Dyed latex beads (Estapor, Merck) were used as detection particles. Specific antibodies were coupled to latex beads following manufacturer indications (EMD Millipore Corporation, 2015). Antibody was added to a final concentration of 0.2 mg mL^{-1} and incubated for 2.5 hours at room temperature with gentle shaking (Rotator, JP Selecta) at 12 rpm and 90° angle. To stop the reaction 30 µL of ethanolamine per mL were added and incubated for 30 min at room temperature. Then, sample was centrifuged (Sigma 1-16K) at 17.000 x g for 15 min and the supernatant was discarded. For blocking beads, BSA at 1% was added and incubated for 2 hours at room temperature with gentle shaking. Quality of the conjugate was assessed by dynamic light scattering (Zetasizer Nano Range, Malvern Instruments).

Conjugates were immediately mixed together at a ratio 1:1:2 (anti-β-lactoglobulin:anti-casein:anti-Internalin A) and dispensed over the conjugate pad (glass fiber membrane, GE) with a ZX 1010 Dispenser (Bio-Dot, Irvine, USA).

2.3 *Preparation of* LFIA strips

The anti-casein antibodies for test line 1 (TL1), anti-β-lactoglobulin for test line 2 (TL2) and recombinant Internalin A for control line (CL), were applied over a nitrocellulose membrane at 1 mg mL^{-1} in 3 independent lines using a ZX 1010 dispenser (Bio-Dot, Irvine, USA) as shown in Figure 1.

To assembly the strip components, nitrocellulose membrane, conjugate pad and adsorbent pads were placed on an adhesive baking card with an overlapping among the components of 2

mm to ensure correct capillary flow. Cards were cut into 4 mm-wide strips with a CM4000 Guillotine Cutter (Bio-Dot, Irvine, USA) and stored with desiccant in closed tubes at room temperature.

2.4 Test procedure

An amount of 1 g or mL of food was mixed with 10 mL of extraction buffer using a filter-plastic bag (BAGPAGE®, Interscience, France). A volume of 150 µL of filtered sample was placed in a test tube. Then, the strip was introduced into the sample and incubated for 10 min. A negative result is obtained when only the control line appears, but none of the test lines. When the control line and one or both test lines appear, the result is considered positive. If the control line is not displayed, the result is considered as invalid.

2.5 *Validation study*

Validation study was performed according to AOAC guidelines. As indicated in Appendix F of the Guidelines for Standard Method Performance Requirements (AOAC International, 2016), the performance evaluation for single laboratory validation of qualitative methods have to include: inclusivity/selectivity, exclusivity/cross-reactivity, environmental interference, laboratory variance and Probability of Detection (POD). POD is the proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level. POD is calculated as the number of positive results divided by the total number of tests at each level of analyte (AOAC International, 2014). Analyses to obtain POD were performed by 3 different analysts, in different days and 4 batches of the test were included in the study. Confidence interval levels, LCL (lower control limit) and UCL (upper control limit) were calculated according to the method described by Wehling, LaBudde, Brunelle, & Nelson, (2011).

2.6 Preparation of samples

2.6.1 POD determination

β-lactoglobulin and sodium caseinate (Sigma-Aldrich) were used to determine the limit of detection for the specific target test lines. Different concentrations of each protein were prepared in extraction buffer and the probability of detection was calculated after analyzing at least 20 independent samples at each level of concentration.

Commercial UHT milk with a protein content of 3.1% was also used as a sample to obtain the limit of detection. The probability of detection at different percentages of UHT milk diluted in extraction buffer was calculated by analyzing at least 20 independent replicates as previously described.

2.6.2 Cross-reactivity study

Thirty-eight food commodities were selected following the recommendations of AOAC guidelines (Abbott et al., 2010). Furthermore, milk form mare, sow, buffalo and camel were also assayed. A minimum quantity of 100 g was mixed thoroughly with a blender in those matrices with a heterogeneous composition.

2.6.3 Confirmation of the limit of detection with spiked and incurred foods

Several foods declared as milk-free were selected to confirm the limit of detection: red wine, herb liquor, soy drink with coffee, Frankfurter sausage, cooked ham, vegetable sauté sauce and soy and rice infant formulas. A minimum quantity of 100 g was mixed thoroughly with a blender in those matrices with a heterogeneous composition. Then, samples were spiked with

0.05% and 1% of UHT milk, except for liquor that was spiked with 0.2% and 1%, to confirm the limit of detection for β-lactoglobulin and casein test lines.

Thermal processed food (bread, sausage and pâté) incurred with 0.1% of commercial non-fat spray dry milk (NFSM) were prepared as described previously (de Luis et al., 2008). Two commercial incurred foods (milk on label), chocolate ice cream and chorizo, were included in the evaluation.

Milk by-products were obtained from the correspondent supplier: WPC 33-Whey Protein Concentrate 33% protein (ILAS, S.A.), total sodium caseinate (Sigma-Aldrich), skim milk powder MQA 0902014 (MoniQA Association) and whole milk powder NIST 1549 (National Institute of Standards and Technology).

Sweet whey was prepared from raw milk obtained from a local farm (Movera, Zaragoza, Spain). Milk was skimmed by centrifuging at 3000xg during 30 min at 4°C. Chymosin from calf stomach (Sigma-Aldrich) was added to skimmed milk and incubated at 37°C for approximately 1 hour. Then, curd and whey was separated by centrifuging at 3000xg for 30 min at room temperature. Acid whey was obtained by adding HCl to raw milk slowly until pH was reduced to 4.6. Whey was separated from precipitated casein by centrifuging at 3000 x g for 30 min.

3 Results& Discussion

3.1 Development of the strip test

To develop a strip test with the ability to detect and identify the two main milk protein fractions (caseins and whey) two target proteins were selected: casein and β-lactoglobulin. Thus, three different detection antibodies were combined into the conjugate pad to produce specific signals in three independent lines, corresponding to control, test 1 for casein and test 2 for β-lactoglobulin.

206 To get an easier interpretation two colored latex particles were combined in the conjugate
207 pad. Red latex particles were coupled to anti-β-lactoglobulin and to anti-casein antibodies to
208 obtain both test lines whereas blue latex beads were coupled to anti-Internalin A antibodies to
209 obtain the control line. Different proportions of the three conjugates were tested to obtain the
210 optimal signal in positive samples without background signal in negative samples. The
211 proportion 1:1:2 was selected since other conditions showed weak signals in low positive
212 control samples or unspecific signals with negative control samples.

213 *3.2 POD concentration study*

214 The limit of detection of the test was determined independently for each one of the milk
215 protein targets in the corresponding test lines. The lowest level of detection with a POD value
216 of 0.95 was 0.5 ppm for β-lactoglobulin (Supplementary material, Table 1) and 2 ppm for
217 caseinate (Supplementary material, Table 2). UHT milk was also analyzed to determine the
218 POD on a thermal processed product in which both targets are present (Supplementary
219 material, Table 3). The UHT treatment was chosen because it is the most common treatment
220 of liquid milk for consumption. Besides, residues of this product could be found in other liquid
221 processed foodstuff when manufacturing lines are shared. It could be also a common
222 contaminant in collective kitchens where it is used for preparing meals instead of milk powder
223 and other dairy by-products. The lowest level of UHT milk that showed a POD of 1.00 was
224 0.05%.

225 The typical overloading or hook effect inherent to this kind of tests was also evaluated. This
226 effect happens when the quantity of the target protein is so high that the binding sites of the
227 antibodies are saturated resulting in a reduction of the analytical color signal and even giving a
228 false negative result. The test line for β-lactoglobulin was not displayed when levels of this
229 protein were higher than 4000 ppm (Supplementary material table 1). When UHT milk was

analyzed without additional dilution, the test line for β-lactoglobulin was visualized but the casein line did not appeared, indicating that the hook effect affected only the later specific line. Although β-lactoglobulin concentration in milk is around 4 g/L (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2014), the lack of hook effect could be attributed to the alteration of some protein epitopes by the heat treatment applied. Our results indicate that the overloading effect could be compensated with the two test lines, due to the different content of milk proteins that can be found in food products. Thus, high content of caseins in a milk powder could produce a hook effect for this milk fraction but not for β-lactoglobulin. Considering the protein content on the certified reference material of milk powder (MoniQA) and the β-lactoglobulin content in powder milk (Bobe, Lindberg, Freeman, & Beitz, 2007), only a concentration higher than 11% (w/w) of milk powder as ingredient in a food sample could result in a false negative for the β-lactoglobulin test line. In turn, the high content of β-lactoglobulin in a WPC could produce a hook effect for this protein but not for caseins.

Previous studies which compared different food allergen methods for milk protein detection highlighted an absence of analytical standardization (Johnson et al., 2014; Török et al., 2015). Following their recommendations, the evaluation of different levels of purified protein targets, whey and casein fractions, as well as non-fat dry milk (NFDM) reference material was compared in our study. Table 1 shows the level of detection of casein and β-lactoglobulin in different dairy by-products widely used in food industry. The test was able to detect 1 ppm of acid whey and 5 ppm of total sodium caseinate, WPC 33, sweet whey and NFDM. As expected, β-lactoglobulin was poorly detected on sodium caseinate with a level of detection of 100 ppm. Recently, it was reported that commercial LFIA tests based on the detection of β-lactoglobulin often fail to detect milk proteins in total sodium caseinate or give a higher level of detection than the tests based on casein detection (Courtney et al., 2016). Test line based on casein detection was able to detect low levels of milk protein in WPC. A similar result was also found with other commercial tests evaluated by Courtney et al. (2016). This is probably due to the

256 fact that some casein molecules are out of the micellar structure and therefore they are
257 released into whey during curdling, being present in WPC. The LFIA test evaluated in the
258 present work showed a lower detection limit for β-lactoglobulin than for casein in whey
259 samples. However, the casein test line was able to detect lower levels of total sodium
260 caseinate and NFDM than the corresponding line for β-lactoglobulin. In addition, the level of
261 detection for β-lactoglobulin was different depending on the source of the NFDM material. The
262 test was able to detect 10 ppm of β-lactoglobulin in MoniQA milk powder reference material
263 whereas the level of detection was increased to 15 ppm when NIST 1549 milk powder was
264 used. This result could be due to the different intensity of thermal processing applied to each
265 product, being the degree of denaturation of β-lactoglobulin higher in NIST than in MoniQA
266 material. Similar results have been reported by other authors when comparing different egg
267 powder certified reference materials (Lacorn, Lindeke, Siebeneicher, & Weiss, 2018).

268 *3.3 Study of cross-reactivity in food commodities*

269 A panel of 38 food commodities based on AOAC recommendations (Abbott et al., 2010) and
270 milk from six different animal sources were analyzed using the developed LFIA test. Despite
271 these food commodities are recommended for ELISA test validation, the same requirement
272 has been established for LFIA test, as long as both tests are applied for the same purposes and
273 based on a similar biochemical principle. No cross-reactivity was found for any of the
274 ingredients analyzed (Table 2). However, in the case of raw meat, seafood and fish the control
275 line displayed a faint signal, indicating some interference with these samples. Several cheese
276 and milk samples from different species were also analyzed to determine the cross-reactivity
277 with bovine proteins. All cheeses made from goat and sheep milk and mozzarella cheese made
278 with water buffalo milk gave a positive result indicating that the test was able to detect milk
279 from these species. However, milks from mare, sow and camel gave a negative result with the
280 test. The reactivity of anti-β-lactoglobulin antibodies with sheep, goat and buffalo milk is

281 expected as the protein share about 94-97% of sequence homology in these species. The
282 absence of reaction with mare and sow milk could be explained by the low homology of β-
283 lactoglobulin of these species with that of bovine milk (less than 60%). Concerning camel milk,
284 the lack of reactivity is due to camelids milk is lacking of β–lactoglobulin. In the case of β-
285 casein, the homology of bovine protein with proteins of sheep, goat and buffalo ranges from
286 91 to 98% whereas with the protein of mare, sow and camelids this percentage is around 60-
287 69% (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2014).

288 *3.4 Confirmation of the limit of detection in food matrices (Matrix study)*

289 It is known that the composition of food matrices could hamper the detection of allergens
290 (Khuda, Jackson, Fu, & Williams, 2015; Poms, Klein, & Anklam, 2004). Therefore, a
291 representative group of food matrices were spiked at least at two levels of added UHT milk
292 (0.05 and 1%). UHT milk was selected as the ingredient for spiking since it is a well
293 standardized product, ease to use and subjected to the most commonly used thermal
294 processing. The lowest level of milk addition (0.05%) was expected to be only detected with
295 the casein test line due to the abundance of this protein in milk, whereas the highest level (1%)
296 would be detected with both, the β-lactoglobulin and casein test lines. The matrices have been
297 chosen not only following the AOAC recommendations (Abbott et al., 2010), but also
298 considering features that could challenge the test. As expected, all the matrices analyzed gave
299 a positive result in both β-lactoglobulin and casein test lines for the level of 1% of UHT milk
300 added (Table 3). Likewise, a positive result was reached at 0.05% of UHT milk in juice, salad
301 dressing, soy drink with coffee, sausage, salad dressing, cooked ham, vegetable sauté sauce,
302 soy infant formula and rice infant formula. However, positive results were only reached at
303 0.2% of UHT milk in red wine and liquor, indicating that ethanol could produce a slight loss of
304 sensitivity. It has been reported that ethanol may hamper or weaken the interaction antigen-
305 antibody (Rehan & Younus, 2006; Singh, Cabello-Villegas, Hutchings, & Mallela, 2010) and thus

306 reduce the ability of antibodies to detect antigens. This fact could be a problem when milk
307 proteins are used as fining agents to clarify alcoholic beverages. However, sample preparation
308 includes a 1/10 dilution that would minimize such issue and in addition new procedures could
309 be adapted for the preparation of this kind of samples. For example, ethanol could be partially
310 evaporated by a soft heating of the samples preventing from its adverse effect. Nevertheless,
311 according to EFSA opinion wines fined with casein may trigger adverse reactions in susceptible
312 individuals and therefore they should be adequately labelled (EFSA Panel on Dietetic Products,
313 Nutrition and Allergies, 2014; Popping & Diaz-Amigo, 2018). Other samples such as soy drink
314 with coffee which contains tannins and polyphenols could be detected at 0.05%, indicating
315 that the extraction buffer was able to neutralize these molecules. This kind of molecules are
316 well known to interfere with the analysis of food allergens by immunochemical techniques
317 (Khuda et al., 2015).

318 *3.5 Incurred food study*

319 Although spiking a food with milk can be used as a model to explore the matrix effect on the
320 results of an analysis, incurred samples are preferable as the effect of the industrial processes
321 on the detection is also evaluated with this kind of samples. Thus, NFDM was added as an
322 ingredient to several foodstuffs before they were processed. The β-lactoglobulin test line only
323 appeared at 10 ppm of NFDM in the sausage, but not in bread or pâté (Table 4). By contrast,
324 the casein was detected at 1 ppm of NFDM in pâté, sausage and bread (Table 4). Pâté and
325 bread were subjected to a thermal process much more intense than sausage, therefore whey
326 proteins such as β-lactoglobulin could have undergone a denaturation degree that hampers its
327 immunodetection. This effect has been previously described by using an ELISA test (de Luis et
328 al., 2009; Monaci, Brohée, Tregoat, & van Hengel, 2011) and mass spectrometry (Lamberti et
329 al., 2016). However, caseins are more resistant to such heat treatments and they could be
330 detected by the LFIA test through the specific line for casein. In a recent study, a LFIA test was

331 developed that could also detect in unique analysis both β-lactoglobulin and casein but
332 samples of non-dairy incurred foods were not assayed (Masiri et al., 2016). Moreover, that test
333 was not able to identify the protein source since antibodies against casein and β-lactoglobulin
334 were mixed in the same test line.

335 The determination of allergens in food using immunoassays has to meet several requirements.
336 The immunochemical technique needs to be highly sensitive in order to detect a low amount
337 of hidden allergen. It also has to be highly specific for the target and not to show cross-
338 reactivity with other food components to avoid false-positive results. Furthermore, another
339 important issue is related to interferences caused by the matrix components, which may
340 hinder extraction of the target analyte resulting in underestimation or false-negative results or
341 which may bind nonspecifically to the antibodies, giving false-positive results (Cucu, Jacxsens,
342 & De Meulenaer, 2013).

343 The effect of processing is probably the main challenge when using immunoassays to
344 determine allergens in foods. Processing usually induce physical and chemical modifications of
345 the target protein that could alter conformational epitopes. Processing may also produce
346 covalent modifications through Maillard reaction or hydrolysis by fermentation that could alter
347 lineal epitopes. Furthermore, processing also often cause protein aggregation that may
348 masked epitopes of the target protein and lose of protein solubility (Gomaa & Boye, 2015;
349 Monaci et al., 2011).

350 The applied extraction buffers and conditions plays a crucial role in the detection of allergens
351 in foods, as only successfully extracted target proteins can be detected by their specific
352 antibodies. The recovery of the extracted target protein depends on the nature of the protein
353 to be analyzed and the degree of denaturation and aggregation induced by processing
354 (Steinhoff, Fischer, & Paschke-Kratzin, 2011).

On the other hand several studies have shown that results obtained by different commercial ELISA tests give incomparable quantitative results when they are used to detect allergenic proteins in processed foods (Gomaa & Boye, 2015; Ivens et al., 2016; Johnson et al., 2014; Monaci et al., 2011). This variability may be explained in part by the use of different target proteins, standards and expression of the reported units as well as to the use of different antibodies and immunoassays formats.

At this respect, one of the great concerns in allergen detection is the lack of certified reference material available. Although recently four testing reference materials for milk allergen has been validated and commercialized (Poms, 2018), the question of how such materials will be detected in different real food matrixes subjected to different processing technologies remain unknown.

3.6 Environmental surface testing

Apart from food matrices, the rinsing water after cleaning could be also analyzed by using the LFIA tests for milk presence in food industry facilities. Thus, some manufacturing plants need to share production lines for several products including some containing milk or its derivatives. In these circumstances, an efficient cleaning procedure becomes essential. A useful checkpoint to control the cleaning efficacy is the rinsing water coming from the Cleaning in Place (CIP) processes (Jackson et al., 2008). The CIP systems usually alternate acid and basic cleaning cycles. Hence, the rinse water could contain residues of the acid or basic products that may affect milk detection. The analysis of water with different concentrations of sodium hydroxide and acid chloride (0.5, 0.25, 0.1 and 0.05N) showed that no interference occurs at or below 0.1N for both products (Supplementary Material Table 4).

3.7 Determination of robustness

The robustness of the method was evaluated by introducing small changes in the standard procedure both for the extraction and the assay itself. Variations in the sample portion to be extracted, extraction buffer volume, volume of sample extract to be analyzed and the temperature of the assay were selected as critical factors. Hence, the effect on sensitivity was evaluated at two levels of UHT milk addition, 1% for the β-lactoglobulin test line and 0.1% for the casein test line. The analysis was performed at least in two independent assays for each condition.

To evaluate the effect of the sample portion, three different quantities (0.8, 1.0 and 1.2 g) of orange juice spiked with UHT milk were mixed with in 10 ml of extraction buffer. Then, the method was performed following the general instructions. In all conditions, a positive result was displayed in the β-lactoglobulin test line for the samples spiked with 1% of UHT milk (Table 5). The casein test line gave a positive result for the samples spiked with both 1% and 0.1% of UHT milk. This result implies that a reduction or increase of 20% in the sample weight neither affected the limit of detection nor caused matrix interference. The same result was found when the variation in the volume of extraction buffer was introduced (8, 10 and 12 mL) with the same sample (Table 5). Thus, a variation of 20% in the volume of the extraction buffer did not affect the detection limit of both test lines.

To evaluate the LFIA method itself, without considering the extraction step, different volumes (0.1, 0.15 and 0.2 mL) of the sample extract were analyzed. Results are summarized in Table 5. For all the conditions, β-lactoglobulin test line gave a positive result for the samples spiked with 1% of UHT milk whereas the casein test line was also positive at 0.1% of added UHT milk. Different assay times were assayed (2-5-10 min) for the same sample (Table 5). Although both test lines were positive at 10 min, they were negative after 2 min and only a faint positive result was shown in the casein test line after 5 min at 0.1% of UHT milk addition. Despite a positive result was reached after 5 min, to obtain suitable results is important to accomplish

403 the assay until 10 min of incubation, according to the manufacturer indications. In addition,
404 the juice sample spiked with 1% of UHT milk gave a negative result for the casein test line and
405 the intensity of the β-lactoglobulin test line was reduced when analyzed at 11°C (result not
406 shown). Therefore, the results from the robustness evaluation pointed out two critical factors
407 to obtain a suitable result, the working temperature and the assay time.

408 *3.8 Product consistency*

409 The lot-to-lot variation was also evaluated and results are summarized in Table 5 of
410 supplementary material. The four batches evaluated were able to detect 0.05% of UHT milk
411 and one reached a level of 0.01%.

412 **4 Conclusions**

413 This work shows for the first time a duplex lateral flow inmunochromatographic assay able to
414 detect simultaneously and independently two different allergenic proteins. In addition to alert
415 for the presence of milk protein residues, the test can identify two of the main allergenic milk
416 proteins (β-lactoglobulin and β-casein). Because both of these proteins are not always present
417 in milk ingredients used in food manufacturing or as processing aids, a method able to
418 simultaneously detect both of them could avoid the choice of only a specific target and would
419 reduce the number of analysis or analytical runs to be performed by users. Thus, these
420 advantages simplify the allergen control and save costs. The in-house validation of the new
421 method has demonstrated a level of detection of 1-5 ppm for whey derivatives through the β-
422 lactoglobulin test line and 1-5 ppm for caseinate and milk powder with the casein test line.
423 Moreover, casein test line showed a better level of detection with thermally processed foods.
424 Method was designed with a blue control line and two red test lines to ease the interpretation
425 of the results. This assay can be applied for milk detection in raw materials, final processed

426 products or to verify the cleaning procedures. It is suitable to be used as a routine tool in food
427 industry without the need for specific training of the staff.

428 **Conflicts of interest**

429 The authors declare no conflict of interest exists.

430 **Acknowledgements**

431 This work has been supported by the Ministerio de Economía y Competitividad (Spain) and by
432 the Fondo Europeo de Desarrollo Regional (European Union). Ref. RTC-2015-3818-2.

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Figure Captions

Figure 1.Scheme of the distribution of the control and test lines in the strip. CL: Control Line, TL1: Test line for casein detection, TL2: Test line for β-lactoglobulin detection.

Highlights

A multiplex LFIA test was developed for simultaneous detection of β-lactoglobulin and casein

Detection of β-lactoglobulin and casein was displayed in independent lines

Limit of detection of 0.5 ppm for β-lactoglobulin and 2 ppm for caseins

The new test was evaluated against incurred food processed matrices.

Table(s)

Table1. Detection of casein and β-lactoglobulin (LGB) in dairy by-products. LGB: β-lactoglobulin. WPC 33: Whey Protein Concentrate 33% protein. NaCas: Total Sodium Caseinate. NFDM: Non Fat Dry Milk[1] Skim milk powder MoniQA reference material. NFDM[2] NIST 1549 Whole milk powder. N: negative, P: Positive.

	WPC 33		Sweet Whey		Acid whey		NaCas		NFDM[1]		NFDM[2]	
	Casein	LGB	Casein	LGB	Casein	LGB	Casein	LGB	Casein	LGB	Casein	LGB
1 ppm	N	N	N	N	N	P	N	N	N	N	N	N
5 ppm	P	P	N	P	N	P	P	N	P	N	P	N
10 ppm	P	P	N	P	N	P	P	N	P	P	P	N
15 ppm	P	P	N	P	N	P	P	N	P	P	P	P
25 ppm	P	P	P	P	N	P	P	N	P	P	P	P
75 ppm	P	P	P	P	N	P	P	N	P	P	P	P
100 ppm	P	P	P	P	P	P	P	P	P	P	P	P

Table(s)

Table 2. Study of cross-reactivity in food commodities. Each sample was analyzed by duplicated. P: positive, P[1]: faint positive, I: invalid, N: negative, LGB: β-lactoglobulin.

Ingredient	Control	Casein	LGB
Almond	P	N	N
Brazil nut	P	N	N
Cashew	P	N	N
Chestnut	P	N	N
Hazelnut	P	N	N
Macadamia nut	P	N	N
Peanut	P	N	N
Pecans	P	N	N
Pine nut	P	N	N
Pistachio	P	N	N
Poppy seed	P	N	N
Pumpkin seed	P	N	N
Sesame	P	N	N
Walnut	P	N	N
Buckwheat	P	N	N
Barley	P	N	N
Corn	P	N	N
Oat	P	N	N
Rye	P	N	N
Wheat	P	N	N
Brown rice	P	N	N
White rice	P	N	N
Chick peas	P	N	N
Lentils	P	N	N
Red beans	P	N	N
Soybean	P	N	N
Split peas	P	N	N
Lupine beans	P	N	N
Cocoa	P	N	N
Coconut	P	N	N
Lecithin	P	N	N
Beef	P[1]	N	N
Chicken	P[1]	N	N
Egg	P	N	N
Pork	P[1]	N	N
Fish	P[1]	N	N
Crustaceans	P[1]	N	N
Gelatin	P	N	N
Camel Milk	P	N	N
Mare Milk	P	N	N
Sow Milk	P	N	N
Buffalo Cheese	P	P	P
Sheep Cheese	P	P	P
Goat Cheese	P	P	P

Table(s)

Table 3. Limit of detection in food matrices spiked with UHT milk. Each sample was analyzed by duplicate in independent assays, except for red wine, liquor and juice which were analyzed by 20 replicates. N: negative, P: Positive, LGB: β-lactoglobulin, POD: Probability of Detection, UCL: upper control limit, LCL: lower control limit, CI: confidence level.

Food Matrix	Spike level	Casein	LGB	POD	LCL (95%CI)	UCL (95%CI)
Orange juice	Blank	N	N			
	1% UHT milk	P	P			
	0.05% UHT milk	P	N	1.00	0.84	1.00
Red wine	Blank	N	N			
	1% UHT milk	P	P			
	0.2% UHT milk	P	N	1.00	0.84	1.00
Liquor	Blank	N	N			
	1% UHT milk	P	P			
	0.2% UHT milk	P	N	1.00	0.84	1.00
Soy drink with coffee	Blank	N	N			
	1% UHT milk	P	P			
	0.05% UHT milk	P	N	1.00	0.84	1.00
Sausage	Blank	N	N			
	1% UHT milk	P	P			
	0.05% UHT milk	P	N			
Salad dressing	Blank	N	N			
	1% UHT milk	P	P			
	0.05% UHT milk	P	N			
Cooked ham	Blank	N	N			
	1% UHT milk	P	P			
	0.05% UHT milk	P	N			
Vegetable sauté sauce	Blank	N	N			
	1% UHT milk	P	P			
	0.05% UHT milk	P	N			
Soy infant formula	Blank	N	N			
	1% UHT milk	P	P			
	0.05% UHT milk	P	N			
Rice infant formula	Blank	N	N			
	1% UHT milk	P	P			
	0.05% UHT milk	P	N			

Table(s)

Table 4. Detection of milk in thermal processed foods incurred with non fat spray dried milk. Each sample was analyzed by triplicate. LGB: β-lactoglobulin. N: negative, P: Positive.

Incurred Matrices	Milk level	Casein	LGB
Pâté	Blank	N	N
	100 ppm NFSM	P	N
	10 ppm NFSM	P	N
	5 ppm NFSM	P	N
	1ppm NFSM	P	N
Bread	Blank	N	N
	100 ppm NFSM	P	P
	10 ppm NFSM	P	N
	5 ppm NSFM	P	N
	1ppm NFSM	P	N
Sausage	Blank	N	N
	100 ppm NFSM	P	P
	10 ppm NFSM	P	P
	5 ppm NSFM	P	N
	1ppm NFSM	P	N
Chocolate ice cream (milk on label)		P	P
Chorizo (milk on label)		P	N

Table(s)

Table 5. Results of the robustness study. Effect of the deviations in the protocol on the sensitivity of the test. Each condition was evaluated by duplicate. LGB: β-lactoglobulin. N: negative, P: Positive. P[1]: faint positive

Deviations in the extraction						
Sample portion						
UHT milk (%)	0.8 g		1 g		1.2 g	
	Casein	LGB	Casein	LGB	Casein	LGB
1	P	P	P	P	P	P
0.1	P	N	P	N	P	P[1]
Extraction buffer volume						
UHT milk (%)	8 mL		10 mL		12 mL	
	Casein	LGB	Casein	LGB	Casein	LGB
1	P	P	P	P	P	P
0.1	P	N	P	N	P	N
Deviations in the assay						
Assay volume						
UHT milk (%)	0.1 mL		0.15 mL		0.2 mL	
	Casein	LGB	Casein	LGB	Casein	LGB
1	P	P	P	P	P	P
0.1	P	N	P	N	P	P[1]
Assay time						
UHT milk (%)	2 min		5 min		10 min	
	Casein	LGB	Casein	LGB	Casein	LGB
1	N	N	P	P	P	P
0.1	N	N	P[1]	N	P	N