

Establishment of a Lateral Flow Colloidal Gold Immunoassay Strip for the Rapid Detection of Soybean Allergen β -Conglycinin

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Abstract A rapid lateral flow colloidal gold immunoassay strip was established in a sandwich format with the colloidal gold-labelled mouse anti- β -conglycinin monoclonal antibody (mAb) and the rabbit anti- β -conglycinin polyclonal antibody (pAb) in order to specifically identify a soybean allergen, β -conglycinin. Soybean and soy derivatives have become ubiquitous in many vegetarian and meat-based food products, and as a result, dietary avoidance has become difficult. Therefore, soybean avoidance is challenging for individuals with soybean allergy. In our study, the selected mAb showed high affinity to the target and no cross-reactivity with other soybean allergens. Based on the sandwich immunoassay principle, the rabbit pAb was applied in the test line with high titre. The assay could be accomplished within 10 min without sophisticated procedures. The limit of detection of the test strip was calculated at 1.66 mg/kg in powdered milk using an optical density scanner that measures relative optical density. The assay showed high specificity for β -conglycinin, with no cross-reactions with other food allergens. The efficacy of the lateral flow test strip to detect β -conglycinin in powdered milk samples ranged between 80.8 and 89.2% with relative standard deviations of less than 7.2%. Therefore, the test strip is useful as a rapid and reliable detection method

for β -conglycinin in powdered milk. In addition, this assay is suitable for monitoring β -conglycinin in other processed foods when samples are appropriately extracted.

Keywords β -Conglycinin · Immunochromatographic strip · Colloidal gold · Immunoassay

Introduction

Soybean (*Glycine max*) is one of the important crops in the world, and it is used extensively due to its nutritional value, physicochemical properties, and health benefits (Friedman and Brandon 2001). However, soybean is included in the “Big Eight” foods identified as the most frequent sources of human food allergens, which are likely responsible for 90% of all food allergies (Sicherer and Sampson 2010). Soybean allergy is very common, affecting approximately 0.4% of young children (Savage et al. 2010). Soybean is a popular vegetarian alternative to meat, and furthermore, many processed foods contain soybean, where its undeclared addition to food presents a potential risk for allergic individuals (Wilson et al. 2005). Soybean allergy can cause adverse physiological symptoms involving the skin, as well as the gastrointestinal and respiratory systems, and may result in hypersensitivity and even death (Wang et al. 2014). According to the EU directive 2007/68/EC, food products containing soybeans and products thereof must be appropriately labelled, regardless of the amount added as an ingredient, in order to protect allergic consumers. Consequently, reliable detection and quantification methods of detecting soybean allergens are necessary to ensure compliance with food labelling requirements and to avoid potential fraud associated with the addition of soybean.

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β -Conglycinin is one of the predominant soybean seed storage proteins, it belongs to the 7S globulin family according to its sedimentation coefficient and accounts for approximately 18.5% of total proteins (Murphy and Resurreccion 1984), and has been demonstrated to be one of the most predominant soybean allergens (Sun et al. 2008). β -Conglycinin causes damage to intestinal morphology by depressing intestinal cell growth, damaging the cytoskeleton, and causing apoptosis in the intestine (Chen et al. 2011), which leads to immune function disorders, growth depression, and diarrhoea. β -Conglycinin has strong thermal stability; thus, the allergen activity cannot be totally destroyed through conventional processing, such as heating. As one of the major soybean allergens, β -conglycinin is a good target for the specific detection of a soybean protein in food and feed.

In recent years, research on soybean allergen detection has rapidly expanded (Wang et al. 2014). Some methods for their detection are based on expensive equipment, such as high-performance liquid chromatography (HPLC) (García et al. 2007) and mass spectrometry (MS) (Houston et al. 2011). Other methods for their detection include polymerase chain reaction (PCR), which is based on the amplification of the specific DNA fragments of allergens (Tian et al. 2011). However, these methods are time-consuming and expensive. Moreover, PCR methods do not detect the allergen itself, and the results are difficult to correlate to actual allergen quantities; thus, it cannot be used in proper risk assessment and management (Cucu et al. 2013). Immunoassays for allergen detection, such as enzyme-linked immunosorbent assay (ELISA) (Chen et al. 2014), which are based on the interaction between a specific antibody and its associated allergen, have been applied due to their high sensitivity and reliability, suitability for high-throughput screening, and relatively inexpensive equipment, but more than 2 h are required to complete such assays.

The immunochromatographic lateral flow test strip a developed technique based on ELISA methods has been considered for allergen detection in recent years because it is inexpensive, rapid, portable, and extremely simple to perform (Koizumi et al. 2014; Peng et al. 2015). Test strips in the sandwich format are becoming a popular diagnostic tool for the detection of high-molecular-mass analytes, such as viruses, bacteria, hormones, and parasite antigens (Qian and Bau 2003). However, these strips are not extensively used for the detection of food allergens, and there are no lateral flow test strips available for the detection of β -conglycinin. Therefore, in this study, we generated a polyclonal antibody (pAb) and a highly specific monoclonal antibody (mAb) against β -conglycinin and developed a novel sandwich format lateral flow test strip for the detection of β -conglycinin in powdered milk standard samples as a useful tool for monitoring food products.

Materials and Methods

Reagents and Materials

Soybean agglutinin (SBA), Kunitz trypsin inhibitor (KTI), peanut agglutinin (PNA), ovalbumin (OVA), casein, Freund's complete/incomplete adjuvant (FCA/FIA), and goat anti-rabbit and goat anti-mouse IgG-horseradish peroxidase (HRP) were obtained from Abbkine (Redlands, CA); nitrocellulose (NC) membranes, glass fibre, and absorbent pads were purchased from Millipore; all other reagents and solvents were of analytical grade or higher. Soybean β -conglycinin and rabbit anti-mouse IgG antibodies used were produced in our laboratory. β -Conglycinin purified according to the procedure described by Nagano et al. (1992), and purity estimated to be 91.7%. Rabbit anti-mouse IgG antibodies were prepared according to the method of Zhang et al. (2009).

In this study, animal immunisations were performed at the Henan Key Laboratory of Animal Immunology (Zhengzhou, China). Eight-week old female BALB/c mice and New Zealand White rabbits were obtained from the Henan Laboratory Animal Center (Zhengzhou, China). All animals were raised and treated according to the principles of the Animal Care and Use Committee of the Henan Academy of Agricultural Sciences.

Preparation of Antibodies

Two New Zealand White rabbits were used to produce pAb against β -conglycinin. Both rabbits were immunised subcutaneously with 200 μ g of purified β -conglycinin. The first injection of immunogen was an emulsion of PBS (0.01 mol/L phosphate-buffered saline, pH 7.4) and FCA. Three booster injections were administered at 3-week intervals with the same dosage of immunogen emulsified in FIA. Three 8-week-old BALB/c female mice were immunised subcutaneously with 50 μ g purified β -conglycinin, followed with the same immune programme as in the rabbit immunisation. The titre of antisera was measured by indirect ELISA, and the anti- β -conglycinin activity was measured by indirect competitive ELISA according to our previously reported steps (Wang et al. 2015). We chose the mouse with the highest anti- β -conglycinin activity for cell fusion. Hybridomas-secreting anti- β -conglycinin mAbs were generated by standard methods (Köhler and Milstein 1975). Selected clones were subcloned by limiting dilution. Ascites were produced in paraffin-primed BALB/c mice, and the mAb was then purified from the antiserum by ammonium sulphate precipitation.

The isotype of the mAb was determined using mouse monoclonal antibody isotyping reagents (Sigma-Aldrich, USA). The measurement of mAb affinity (K_a) was performed

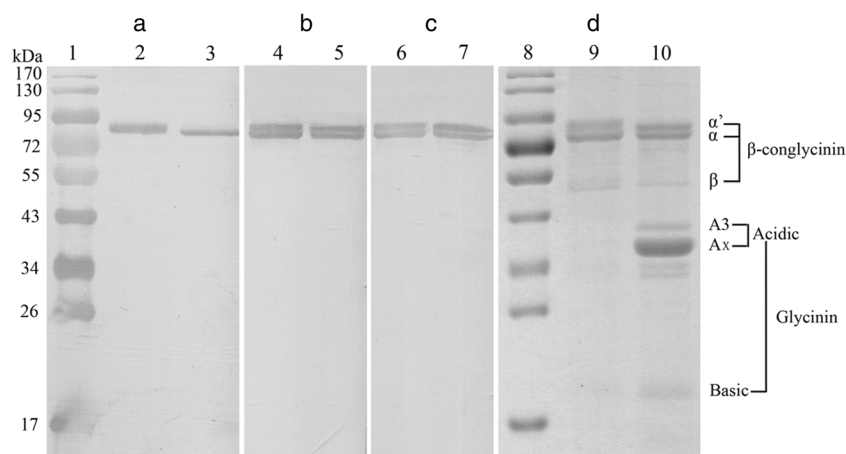


Fig. 1 Western blot analysis of mAb with purified β -conglycinin and soybean protein isolate. **a** mAb 1G6. **b** mAb 7E7. **c** mAb 8C11. **d** SDS-PAGE. Lanes 1 and 8: pre-stained protein marker. Lanes 2, 4, 6, and 9:

purified β -conglycinin. Lanes 3, 5, 7, and 10: soybean protein isolate. Subunits of β -conglycinin (α' , α , and β) and polypeptide chains of glycine are shown on the right (Ax indicates A1, A2, and A4)

according to the procedure described by Beatty et al. (1987). The specificity of mAb was assayed using Western blot according to our previously reported steps (Wang et al. 2015).

Preparation of Test Strips

Colloidal gold was produced by reduction of gold chloride with 1% sodium citrate (w/v), as described previously (Zhang et al. 2006). Approximately 20 μ L of purified mAb (16 mg/mL) was added to 10 mL of the colloidal gold solution with constant stirring for 30 min. Then, 1 mL of 0.02 mol/L sodium borate (pH 9.0) containing 10% (w/v) BSA was added while stirring, and the mixture was incubated at room temperature for 10 min in order to stabilise the conjugate. The labelled mAb was washed with wash buffer [0.02 mol/L sodium borate, pH 9.0; 1% (w/v) BSA] by repeated centrifugation at 25,000 g and 10 $^{\circ}$ C for 30 min. The precipitate was resuspended in 1 mL of resuspension solution [0.02 mol/L sodium borate, pH 9.0; 1% (w/v) BSA, 3% (w/v) sucrose, and 0.03% (w/v) sodium azide] and stored at 4 $^{\circ}$ C before use for the preparation of conjugation pad. The test strips were fabricated according to the previously reported steps (Wang et al. 2015).

Test Procedure and Principle

One piece of the test strip is dropped with 100 μ L of standard samples or sample extract solution into the sample pad, and then the solution migrates and reacts with labelled mAb. It takes approximately 10 min to complete a test. If the sample is positive, β -conglycinin binds to the labelled mAb first and then binds to pAb, thus forming a red band in the test line. Higher amounts of β -conglycinin in the sample result in a

stronger test line. On the contrary, if the sample is negative, there is not a red band in the test line. To serve as a procedural control, a red band always appears in the control line regardless of β -conglycinin presence.

Evaluation of the Test Strips

The sensitivity of the test strips was determined by testing a series of diluted standard samples containing various concentrations of purified β -conglycinin at 0, 50, 100, 200, 400, 800, 1600, 3200, 6400, and 12,800 ng/mL. The assays were carried out in triplicate by using the test strips. Test lines were scanned with TSR3000 Membrane Strip Reader (BioDot, USA) to obtain the relative optical density (ROD). The standard curve was constructed by plotting the $G/D \times \text{area}$ (graph density $\times$ area)—ROD percentage obtained from the standard samples against the logarithmic concentrations. $G/D \times \text{area}$ —

Table 1 Average $G/D \times \text{area}$ —ROD of the test lines of the series of diluted standard samples

β -Conglycinin concentration (ng/mL)	$G/D \times \text{area}$ —ROD (pixel)
0	1.7456
50	4.1973
100	7.1879
200	16.8506
400	34.5664
800	64.3391
1600	89.1198
3200	115.0264
6400	131.2944
12,800	129.8362

ROD percentage was $G/D \times \text{area-ROD}$ of the standard sample divided by the $G/D \times \text{area-ROD}$ at saturation, meaning the binding degree of β -conglycinin with test line. The linearity of the method was assessed by the coefficient of determination (R^2), and the limit of detection (LOD) was calculated from the regression equation. The specificity of the test strips was identified by testing two soybean allergens (SBA, KTI) and other food allergens (PNA, OVA, and casein) with a high concentration (20 $\mu\text{g/mL}$).

We procured commercially powdered milk without soybean included in the list of ingredients, which was used in this study to evaluate β -conglycinin recovery by the strip test. Powdered milk samples were spiked with purified

β -conglycinin at 3, 9, and 27 mg/kg, and samples were subjected to the defatting procedure according to the method of Hei et al. (2012). Protein was then extracted by making a slurry with a 15-fold volume of water (adjusted to pH 9.0 with 2 N NaOH) under continuous agitation for 1 h, followed by centrifugation at 9000 g for 30 min; the supernatant was then filtered through a 0.45- μm Millex GP filter (Millipore, Cork, Ireland). The protein supernatants were analysed by the test strips. Intra-assay accuracy was estimated using one batch of the test strips. For inter-assay accuracy, three batches of the test strips were used to test the samples. The recovery and coefficient of variation (CV, %) were calculated from triplicate assays in all cases.

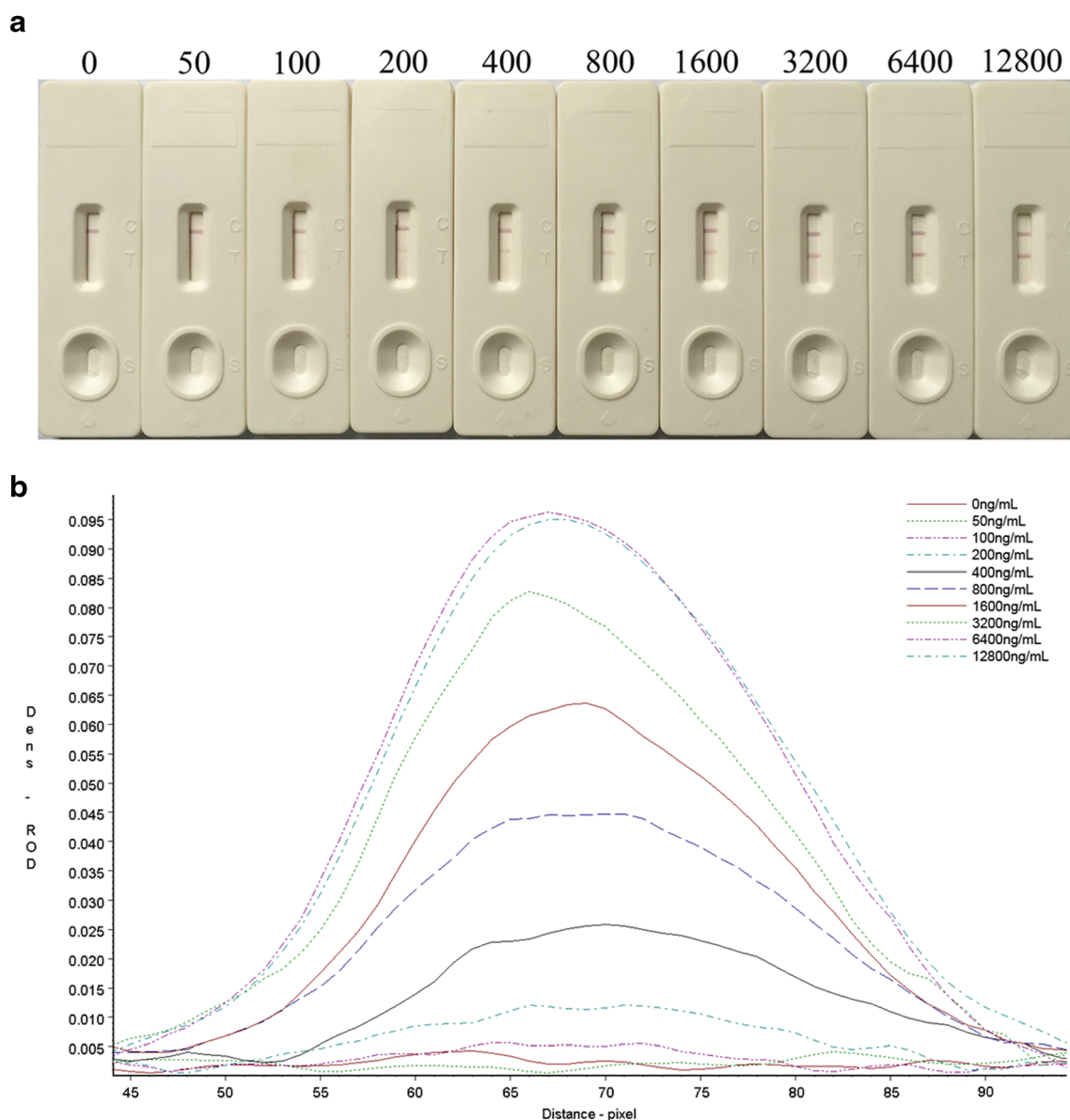


Fig. 2 **a** Sample extracts added with β -conglycinin standard solutions were tested using the test strips. **b** ROD curves of the standard samples at 0, 50, 100, 200, 400, 800, 1600, 3200, 6400, and 12,800 ng/mL were tested using the test strips

Results and Discussion

Characterisation of Antibodies

The pAb from rabbit no. 1 was selected for further study due to its high titre of $1:5.12 \times 10^5$, which was the highest titre among the pAbs produced. After cell fusion and screening, three more sensitive hybridomas, named 1G6, 7E7, and 8C11, were intraperitoneally injected into mice in order to produce ascites, and then the mAbs were purified from the ascites by ammonium sulphate precipitation. The titres of 1G6, 7E7, and 8C11 were $1:2.56 \times 10^5$, $1:1.024 \times 10^6$, and $1:5.12 \times 10^5$, respectively; the affinity constants (K_a) of 1G6, 7E7, and 8C11 were 3.29×10^8 , 1.34×10^9 , and 3.48×10^8 L/mol, respectively. The isotypes of the mAbs were identified as IgG1. The mAb 7E7, having both the highest titre and the highest affinity, was chosen to establish the lateral flow strip.

Western blot results showed that mAb 1G6 has a single reacted band, corresponding to the α' subunit of β -conglycinin (Fig. 1a), but mAb 7E7 and 8C11 both reacted with α' and α subunits of β -conglycinin (Fig. 1b, c), possibly due to the high homology (90.4%) in the core region's amino acid sequences between the α' subunit and the α subunit (Maruyama et al. 1998). These results indicated that the mAbs reacted with β -conglycinin specifically without cross-reaction with other soybean proteins.

Sensitivity of the Test Strip

The sensitivity of the test strip was determined by testing the series of diluted standard samples. The $G/D \times \text{area-ROD}$ values increased as the β -conglycinin concentration in the samples increased, but they were saturated above 6400 ng/mL (Table 1 and Fig. 2).

Plotting the concentration of standard β -conglycinin against the $G/D \times \text{area-ROD}$ produced a sigmoidal dose-response curve, which showed good linearity over the range of 100–6400 ng/mL. The standard curve was constructed by plotting the $G/D \times \text{area-ROD}$ percentage obtained from the standard samples against the logarithmic concentrations (Fig. 3). With the scanner, the LOD was quantitatively defined as the amount of β -conglycinin in the standard sample solution that caused a 10% increase in the $G/D \times \text{area-ROD}$ percentage produced by the 0 ng/mL β -conglycinin sample. Thus, the LOD for the test strip was calculated to be a β -conglycinin concentration of 110.46 ng/mL (equivalent to 1.66 mg/kg) on powdered milk samples.

Specificity of the Test Strip

SBA, KTI, PNA, OVA, and casein, each at a concentration of 20 $\mu\text{g/mL}$, were tested by the strips (Fig. 4). The colours of the test lines were the same as that of the negative sample. These

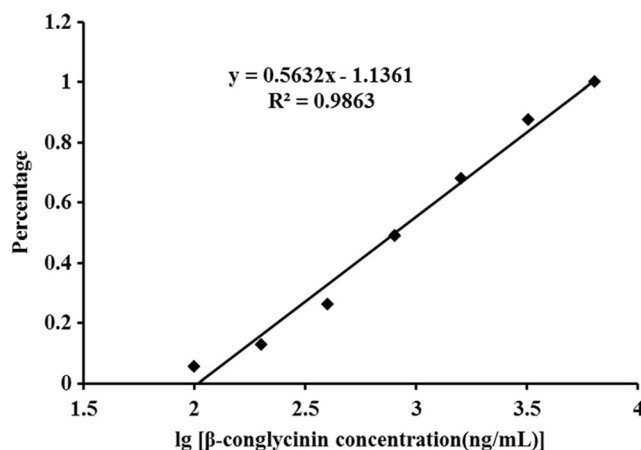


Fig. 3 Standard curve for β -conglycinin using test strip detection. The X-axis represents the logarithmic concentration. The Y-axis represents the percentage of $G/D \times \text{area-ROD}$ of the standards divided by the $G/D \times \text{area-ROD}$ at saturation

results demonstrated that the test strip for β -conglycinin was highly specific and showed negligible cross-reactivity with other food allergens.

The key requirement for a successful strip test is the antibody with high specificity and affinity (Dzantiev et al. 2014). In the present study, the monoclonal antibody prepared by the selected clone, 7E7, showed particularly high affinity for β -conglycinin and showed no cross-reactivity with other soybean proteins, resulting in a high specificity for β -conglycinin.

Recovery of β -Conglycinin in Powdered Milk Sample

The test was performed in triplicate with a single batch of test strips. The optical density of the test line was measured using the test strip reader, and sample values were calculated from the standard curve. As shown in Table 2, the recoveries ranged

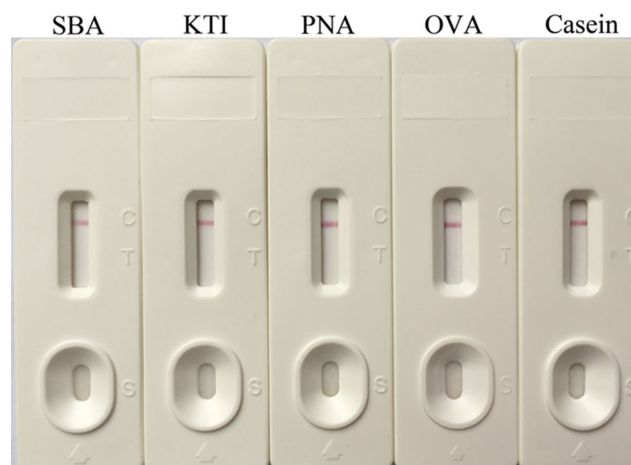


Fig. 4 Soybean agglutinin, trypsin inhibitor, PNA, OVA, and casein, each at a concentration of 20 $\mu\text{g/mL}$, were tested by test strip detection

Table 2 Recovery and intra- and inter-assay accuracy of the test strips for β -conglycinin-spiked powdered milk samples

Spiked β -conglycinin (mg/kg)	Intra-assay			Inter-assay		
	Mean $\pm$ SD (mg/kg)	Mean recovery (%)	CV (%)	Mean $\pm$ SD (mg/kg)	Mean recovery (%)	CV (%)
3	2.43 $\pm$ 0.16	80.8	6.6	2.45 $\pm$ 0.18	81.5	7.2
9	7.65 $\pm$ 0.3	84.9	3.9	7.51 $\pm$ 0.36	83.4	4.8
27	24.09 $\pm$ 1.05	89.2	4.3	23.4 $\pm$ 1.31	86.7	5.6

from 80.8 to 89.2%, with the highest coefficient of variation (CV) occurring at 7.2%, which clearly meets analytical requirements.

Conclusions

In conclusion, we established and evaluated a novel lateral flow colloidal gold immunoassay strip for the rapid detection of soybean allergen β -conglycinin. The lateral flow test strip resulted in a highly sensitive test with a LOD of 1.66 mg/kg for β -conglycinin, which is sufficient for the determination of β -conglycinin in powdered milk. Because the test strip is rapid, simple, reliable, and highly specific, this assay is suitable for monitoring β -conglycinin in other processed foods when samples are appropriately extracted.

Compliance with Ethical Standards

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Conflict of Interest Yao Wang declares that he has no conflict of interest. Zhaozhou Li declares that he has no conflict of interest. Yafeng Pei declares that he has no conflict of interest. Qingmei Li declares that he has no conflict of interest. Yaning Sun declares that he has no conflict of interest. Jifei Yang declares that he has no conflict of interest. Yanyan Yang declares that he has no conflict of interest. Yubao Zhi declares that he has no conflict of interest. Ruiguang Deng declares that he has no conflict of interest. Yuze Hou declares that he has no conflict of interest. Xiaofei Hu declares that he has no conflict of interest.

Ethical Approval Henan Academy of Agricultural Sciences guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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