Potravinarstvo, vol. 7, 2013, no. 1, p. 186-190
doi:10.5219/311
Received: 11 November 2013. Accepted: 11 November 2013.
Available online: 19 December 2013 at www.potravinarstvo.com

ISSN 1337-0960 (online)

COMPARISON OF THE SENSITIVITY OF DETERMINING SOYBEAN ALLERGENS BY ELISA METHOD AND SYBR GREEN I

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ABSTRACT

The aim of this study was to compare the suitability of two methods for detecting defatted soybean powder; SYBR Green I Real-time PCR and enzyme-linked immunosorbent assay (ELISA). Analysis of 20 artificially contaminated samples prepared by simple dilution with wheat flour revealed that both techniques were able to detect defatted soybean powder, although there were significant differences between the two methods. Wheat flour contamination with defatted soybean powder was detected in samples 1-5, (0.012 %; 120 mg.kg^{-1}), but not in samples with lower contamination with soybean powder saples 6-20 using SYBR Green I real-time PCR. Samples 1-10 could not be quantified by ELISA as the absorbance values were greater than the detection limit, and while samples 11-20 were measured, only the values of samples 16, 17 and 18 were within the guaranteed quantification range specified by the ELISA kit manufacturer. Defatted soybean powder contamination was detected in samples 19 and 20, but absorbance values were highly similar to those of the negative control sample.

Keywords: ELISA; PCR; soybean; allergen; SYBR Green I

INTRODUCTION

Allergic reactions to foods are an important medical problem throughout the industrialized world. The occurrence of food allergy appears to be strongly influenced by genetic background of an individual, but the basis of the genetic predisposition to food allergy has not been differentiated from that for atopy in general. In addition, genetic susceptibility alone does not explain the prevalence of food allergy satisfactorily, it is important to consider the importance of environmental influences (external, maternal, and gastrointestinal environment) and interactions between the host and the environment (**Dreskin, 2006)**.

Food allergy is characterized by acute reactions such as the oral allergy syndrome, urticaria, angioedema, vomiting, diarrhea, dyspnea, allergic asthma, bronchospasm, dermatitis, edema, eczema, rhinitis and even anaphylactic shock **(Flinterman et al., 2006)**. Anaphylaxis is a serious, rapid-onset, allergic reaction that may cause death. Severe anaphylaxis is characterised by life-threatening upper airway obstruction, bronchospasm and/or hypotension. Anaphylaxis in children is most often caused by food **(Brown et al., 2006)**. Delayed reactions such as flare up of eczema may occur, but are less frequently reported **(Heine et al., 2002, Novembre a Vierucci, 2001)**.

Many tests for the detection of soy proteins in foods have been described in the literature. Their efficiency depends mostly on the type of food product and detection tool. Six methods were compared using eight food products by **Pedersen et al., (2008)**.

A sandwich ELISA aimed at native soy proteins had the lowest detection limit, but only in a limited number of products. PCR methods have different detection limits, but are useful for all products. Their advantages also include simultaneous detection of genetically-modified (GM) products and sensitivity for highly processed food **(Zhang et al., 2007)**. Using of well defined DNA based markers in species identification is a very useful tool for PCR and resticton clevadge based methods for food and foodstufs analyses **(Žiarovská et al., 2013)** and **(Žiarovská and Poláčeková, 2012)**.

MATERIAL AND METHODOLOGY

Detection of defatted soybean powder frequently used for commercial food production has been performed using two methodologies, Elisa method and SYBR Green qPCR method. SYBR Green qPCR method detect DNA that is resistant to food processing, whereas ELISA methods detect soy protein and also permit quantitation. The two general approaches were used for analysis of artificially contaminated samples prepared by simple dilution by wheat flour (Table 1). Sample one was prepared as a combination of 1 g of defatted soybean powder with 1 g of wheat flour that was then homogenized by vortexing. The next sample was prepared as combination of 1 g of the previous sample and 1 g of wheat flour, and this procedure was repeated until preparing all of 20 samples. Laboratory analysis of the samples was performed using two detection methods: ELISA (standard) and SYBR Green I real-time PCR.

ELISA method

Soy flour was quantified using the Veratox For Soy Flour Allergen Test (Neogen Corp., Lasing, MI, USA). Samples were prepared according to accepted sampling techniques (Neogen food allergen handbook). This involved preheating samples to 60 °C by immersing the bottle containing the extraction solution in a water bath. The samples (1 g) were transferred into 50 mL plastic tubes, together with an extraction additive (0.2 g). The extraction solution (25 mL) was poured into plastic sample tubes that were capped and shaken in a water bath at 60 °C for 15 min. After extraction, the plastic tubes were removed from the bath and material was settled for 5 min before the next step. The extracts were filtered by pouring at least 5 mL through a filter and the filtrates as the samples were collected. The liquid lying above a solid residue after crystallization, precipitation, centrifugation, or other process. The filtrate was used as the samples. The extracts were cooled to room temperature (18-30 °C) prior to analysis.

Twenty red-marked mixing wells for samples and five red-marked wells for controls were removed and placed in the well holder. An equal number of antibody-coated wells were removed and the strip was placed in the well holder. Control and sample extracts (150 µL) were transferred to the red-marked mixing wells using a new pipette tip for each sample. The controls and sample extracts (100 µL) were transferred to the antibody-coated wells using a 12-channel pipette, then wells were mixed in the well holder for 20 s before being incubated for 10 min at room temperature. The content of the well was emptied, and each antibody well was washed using a wash bottle filled with wash buffer, then the solution was discarded. This procedure was repeated 5 times and then the wells were inverted and tapped out on a paper towel to remove the washing solution. The wells were incubated for 10 min at room temperature, then washed with the wash buffer solution as described previously. Conjugate from the green-labeled bottle (100 µL) was transferred into the wells using a 12-channel pipette, and then the well holder was mixed for 20 s and incubated for 10 min at room temperature. The red stop solution from the red-labeled bottle (100 µL) was transferred into all the wells using a 12-channel pipette, then samples were mixed in the well holder for 20 s. The bottoms of the wells were wiped and the absorbance at 650 nm was measured using a Stat Fax 321 Plus microwell reader (Awareness Technology, Palm City, FL).

SYBR Green I real-time PCR

Primers were designed in accordance with **Tengel et al. (2001)**. Primers:
LE1 (5'-GACGCTATTGTGACCTCCTC-3')
LE2 (5'GAAAGTGTCAAGCTTAACAGCGACG-3')
amplified a 318 bp stretch of the soybean (*Glycine max*) lectin gene. Reactions consisted of 12.5 µL of 2x SYBR Green I Hot Start Real-Time PCR Mix (Ecoli Ltd., Bratislava, Slovakia), 500nM of each primer and 2 µL of template DNA at a final concentration of 50 ng.µL^{-1}. The reaction solution was supplemented with double distilled water to a total volume of 25 µL. PCR conditions consisted of a single pre-denaturation step at 95 °C for 1 min, followed by 40 cycles of the profile: 15 s at 95 °C; 20 s at 60 °C; 25 s at 72 °C; 2 s at 82 °C followed by measurement of fluorescence. Final extension was performed at 72 °C for 5 min. The melting curve of PCR products was determined by heating samples to 95 °C, then immediately cooling to 65 °C for 15 s. Samples were heated at a rate of 0.1 °C/s and with each change in temperature of one tenth of a degree the fluorescence was linear. PCR reactions were performed in a LightCycler 1.5 capillary cycler using v4.05 software (Roche).

Table 1 Contamination of wheat flour by defatted soybean powder

Soybean contamination		
sample	%	mg.kg^{-1}
1	50	-
2	25	-
3	12.50	-
4	6.25	-
5	3.13	-
6	1.56	-
7	0.78	-
8	0.39	-
9	0.20	-
10	0.10	-
11	0.050	488.30
12	0.024	244.10
13	0.012	122.10
14	0.0061	61.0
15	0.0031	30.50
16	0,0015	15.30
17	0.0008	7.60
18	0.0004	3.80
19	0.0002	1.90
20	0.0001	1.0

RESULTS AND DISCUSSION

Two different experimental approaches were used to analyze 20 samples of wheat flour that were intentionally contaminated with defatted soybean powder in amounts ranging from 50% to 0.0001% (Table 2).

Results obtained using the ELISA method

In accordance with the range of the assay described by the manufacturer, the ELISA was capable of measuring soy contamination in the range of 2.5 to 25 ppm ($mg.kg^{-1}$) of soy protein. We obtained a correlation coefficient (R2) value of 0.999, which is evidence of the linearity of the assay. Results obtained using the ELISA method are presented in Table 2.

For samples 1-10, the percentage of actual contamination with defatted soy flour could not be quantified since the absorbance values were greater than the detection limit of the assay. Samples 11-20 were successfully quantified, but only the measured values of samples 16, 17 and 18 can be considered correct. These correspond to the values 15.30 ppm, 4.90 ppm and 3.20 ppm of soy protein that are within the guaranteed range of the kit as indicated by the manufacturer. The measured values of samples 15 (30.10 ppm) and 19 (0.90 ppm) were outside of the guaranteed range of quantification and should be disregarded due to significant differences between the measured values and the maximum and minimum limits of the kit. The reported lower limit of the assay is greater than the value of 1 ppm defined by **Koppelman et al., (2004)**. They detected soy in following test materials: native soybean meal, soy protein isolate, soy protein concentrate, and textured soy flakes with using sandwich and inhibition ELISAs. A competition ELISA format resulted in a sensitive test with a detection limit of 0.02 g/ml, corresponding to 0.4 ($mg.kg^{-1}$) 0.4 ppm) in food samples.

Espineira et al. (2010) used an experimental approach similar to ours to determine the detection sensitivity of DNA-targeted methods (end-point PCR and TaqMan real-time PCR) with comparison to reference values for soy protein obtained using an ELISA, and they reported a much higher detection limit of ~5000 $g.kg^{-1}$. This value indicates a comparatively lower sensitivity of the ELISA relative to the range of 2.5 $mg.kg^{-1}$ - 25 $mg.kg^{-1}$ that was validated experimentally in the present study. The values reported herein are close to the manufacturers' guaranteed lower limit of quantification. They are also comparable to those of **L'Hocine et al. (2007)**, who evaluated the effectiveness of commercially-available ELISA kits for the detection of soy in selected food commodities and achieved detection limits of ~2 ppm and <1 ppm with two different assays.

Green I real-time PCR method

As shown in Figure 1, the fluorescent signals obtained by SYBR Green real-time qPCR generated smooth curves that were positively correlated with the cycle number and the concentration of defatted soy flour in the range of 0.78% to 0.0004%. The contamination of 0.10% soy in the sample was the threshold cycle at which the fluorescence intensity exceeded the level of non-specific background.

Samples with contamination of 0.0061% to 0.0004% soy flour showed only very small differences in threshold cycle value in excess of the non-specific background reactions, as well as minimal differences in the shape of the fluorescence curves. For these reasons, it was not possible to determine with precision the threshold of the number of cycles for the various samples by carrying out an assessment of the fluorescent curves, nor was it possible to clearly distinguish the individual exponential and linear phases of the curves.

Table 2 Result of detection by ELISA and SYBR GREEN I (+ positive detection - impossible to detect)

Soybean contamination			ELISA		SYBR Green I
sample	%	$mg.kg^{-1}$	$mg.kg^{-1}$		
1	50	-	Out of detection limit	Out of quantification range	+
2	25	-			+
3	12.50	-			+
4	6.25	-			+
5	3.13	-			+
6	1.56	-			-
7	0.78	-			-
8	0.39	-			-
9	0.20	-			-
10	0.10	-			-
11	0.050	488.30	756.90		-
12	0,024	244,10	251.30		-
13	0.012	122.10	86.30		-
14	0.0061	61.0	43.10		-
15	0.0031	30.50	30.10		-
16	0.0015	15.30	15.30		-
17	0.0008	7.60	4.90		-
18	0.0004	3.80	3.20		-
19	0.0002	1.90	0.90		-
20	0.0001	1.0	0.60		-

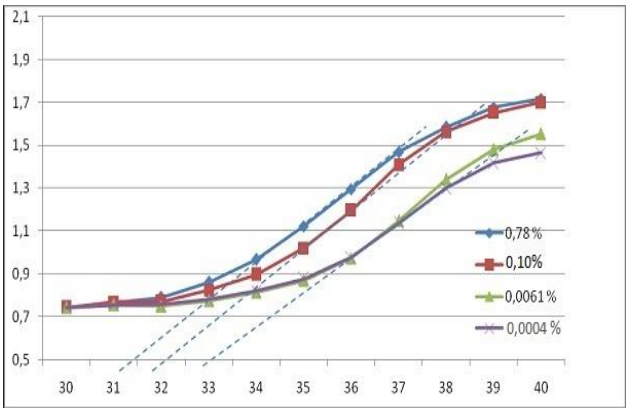

Figure 1 The curve of fluorescence in samples with different concentrations of defatted soy flour

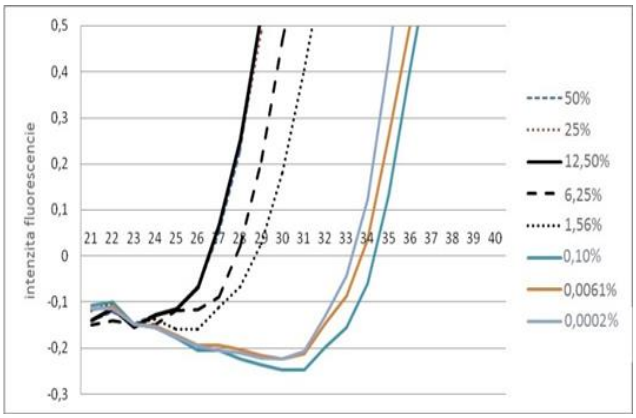

Figure 3 The Analysis of the increase in fluorescence in samples with different concentrations of defatted soy flour

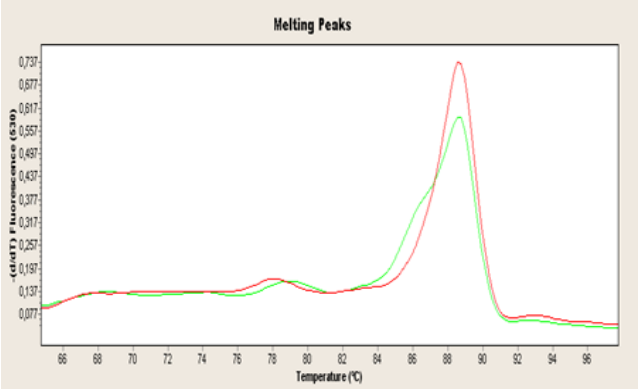

Figure 2 Melting curve of PCR products for samples with a concentration of 0.78% (green linea) and 0.10% (red line) defatted soy flour

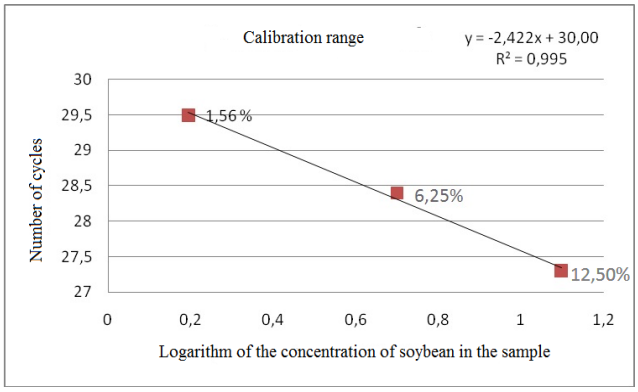

Figure 4 The calibration range for detection of defatted soy flour

Melting curve analysis showed that the PCR product obtained in the 0.10% defatted soy flour sample consisted of a single discrete fragment with a melting point of 88.5 °C (Figure 2).

Moreover, from the figure it is apparent there was little difference in fluorescence (melting peaks) between samples, a finding that points to the specificity of the selected primers and the amplification of only one DNA fragment specific for soybean.

Analysis of the increase in fluorescence (Figure 3) shows comparable values for samples containing 50%, 25% and 12.50% soybean flour.

These samples were, therefore, not used as a basis for constructing the calibration curve since the course of their fluorescence curves was not a direct reflection of the amount of soy in the sample. Furthermore, it was not possible to discriminate differences in the various stages of the fluorescence curves of the PCR products. Similar results were obtained at relatively low proportions of soybean, including 0.10%, 0.0061% and 0.0002%. For these samples, changes in the fluorescence curves were noted during the transition from the linear to the exponential phase, but subsequent melting curve analysis and agarose gel electrophoresis of the PCR products confirmed these were non-specific. Similarly, in evaluating the fluorescence curves of samples with 0.10% and 0.0002% soybean flour it was noted that the curve of the 0.0002% sample was above background (but still non-specific), although this was not the case for the 0.10% sample. Analysis of the curves proved the relationship between fluorescence intensity and the linear phase of PCR amplification, but only in samples composed of 12.50%, 6.25% and 1.56% soybean.

As shown in Figure 4, in the range of 1.56% - 12.50% soybean flour it was possible to quantify the presence of soy by linear regression with high reliability (R^2 = 0.995). Samples outside this range could not be quantified due to the non-linear relationship between fluorescence intensity and analyte concentration, or due to the non-specificity of the PCR product obtained. The sensitivity range of our assay was lower than that reported by **Karudapuram a Batey (2008)**, who used a real-time qPCR protocol for detection and quantification of GM soybeans. The authors achieved a linear range extending from 0.5% - 5% GM soy standard, with a correlation coefficient of 0.992.

Hernández et al., (2003), developed a melting curve-based SYBR Green I multiplex qPCR assay to detect GM soybean that achieved a sensitivity of 0.10% and was comparable to our methodological approach.

Wang, & Fang (2005), performed a quantitative analysis of samples with 20%, 10%, 5% and 1% containing GM soya and 5% - of GM soya using standard multiplex real-time PCR with SYBR Green I with a correlation coefficient of 0.9683.

CONCLUSION

We were able to detect the presence of defatted soybean powder in artificially contaminated samples using both analytical methods. Contamination of wheat flour with defatted soybean powder was detected up to sample 5 (0.012 %; 122 mg.kg^{-1}) using a SYBR Green I real-time PCR method, but not in more dilute samples 6-20. The soy content of samples 1-10 could not be quantified by ELISA because the absorbance values were above the detection limit. Samples 11-20 were quantified, but only the values obtained for samples 16, 17 and 18 were within the guaranteed quantification range of the ELISA manufacturer. We detected defatted soybean powder contamination in samples 19 and 20, but the absorbance values were highly similar to those of the negative control sample.

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Acknowledgements:

This work was supported by grant VEGA 1/1074/11.

This project is being co-financed by the European Union, ITMS 26240220080. We support research activities in Slovakia

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