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Detection of soy in food from the Czech market using ELISA and PCR methods

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Abstract: Soy is considered an allergen under Regulation No. 1169/2011 of the European Parliament and of the Council, which mandates the labelling of soy allergen on food packaging. This study is focused on detecting soy in food products using two kinds of methods. The first method, enzyme-linked immunosorbent assay (ELISA), was performed using two commercial kits (Veratox for Soy Allergen; Neogen, USA). The second method, polymerase chain reaction (PCR), was carried out with two different deoxyribonucleic acid (DNA)-isolating kits and DNA isolation via the cetyltrimethylammonium bromide (CTAB) method. A total of 57 samples of food were tested, including 45 samples of animal origin and 12 samples of plant origin. The results were compared with information on the packaging. From the group of samples that contained soy according to the packaging (12 pieces), 9 samples were found to be positive by ELISA method, 10 samples by the CTAB method and 11 samples by GeneSpin. On the other hand, from the group of samples that should not contain soy according to the packaging (30 pieces), the presence of soy in 2 samples was detected by ELISA. No significant difference was found between the examined methods. Results show that the situation on the market is satisfactory and that only products declared as containing traces of soy appear to be problematic.

Keywords: allergen; processed product; product labelling; Regulation No. 1169/2011; CTAB method

In accordance with Regulation (EC) No. 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, soy and products containing soy are classified as substances or products that cause allergies or intolerances. In accordance with Article 21, these substances, listed in Annex II to this Regulation, must then be highlighted on respective food packaging in such a way as to be clearly distinguishable from the other ingredients in the list of ingredients, for example, by means of type or font or background colour. Rudy and Dreusch (2009) state that epidemiology studies have shown that about 20% of the population of industrialised countries suffer from allergies, and this number is increasing. A meta-analysis of studies has found the incidence of self-reported food allergies ranges between 3% and 35% (Mills 2007), and Poms et al. (2004) stated that food allergies affect up to 2% of the adult population and up to 8% of children. Therefore, it is very important to indicate accurate information about food on the packaging. It is the responsibility of the food business operator to provide this information. Sometimes, however, this information is not provided, which can be misleading and problematic for allergy sufferers. According to DunnGalvin et al. (2015), precautionary allergen labelling (PAL) was introduced by the food industry to help manage and communicate the possibility of reaction from the unintended presence of allergens

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in foods. According to Regulation (EC) No. 1169/2011, the use of PAL should indicate the possible, unintended presence of an allergen in a consumed portion of a food product at or above any proposed action level. Although not directly applicable to PAL, these provisions must be taken into account when considering how to provide information on the possible and unintentional presence in food of substances or products causing allergies or intolerances with the overall aim to ensure clear, meaningful and consistent information to consumers. DunnGalvin et al. (2015) showed that in the current form, PAL is counterproductive for consumers with food allergies. The lack of agreed reference doses has resulted in inconsistent application of PAL by the food industry and in levels of contamination.

So, information on the possible and unintentional presence in food of substances or products causing allergies or intolerances is essential. In the Czech Republic, in the supervisory activities, the Czech Agriculture and Food Inspection Authority and the State Veterinary Administration in Prague when performing the supervisory activities consider traces of soy allergen at concentrations up to 25 mg kg^{-1} (Czech Agriculture and Food Inspection Authority 2018). Concentration up to 2.5 mg kg^{-1} is considered as zero values. Padua et al. (2019) stated that the number of notifications for soy has increased in the last three years in comparison with the period from 2012 to 2014, but this increase is not significant.

Several methods can be used to detect soy in food, including enzyme-linked immunosorbent assay (ELISA) (Scharf et al. 2013) and polymerase chain reaction (PCR) (Stefanova et al. 2013) with isolation using cetyltrimethylammonium bromide (CTAB), as well as microscopic, histological, and immunohistochemical methods (Pospiech et al. 2011) and chromatographic methods (Leitner et al. 2006). Deoxyribonucleic acid (DNA)-based methods (like PCR techniques) are methods of choice due to their specificity and sensitivity and are suitable for the detection of very small amounts of DNA in processed food products (Mafra et al. 2008; Jasbeer et al. 2010). ELISA is the official method for the detection and quantification of allergens in food. Using ELISA, allergens or specific markers of proteins can be detected by a colourimetric reaction following binding with a specific enzyme-labelled antibody (Poms et al. 2004). Planque et al. (2016) showed that high-performance liquid chromatography combined with mass spectrometry for the analysis of food allergens is becoming increasingly interesting and attractive. Its specificity and sensitivity allow it to be applied to processed and raw foods, and it can be used to detect several allergens simultaneously.

This study was focused on the assessment of two methods of soy detection in food (PCR and ELISA) in combination with three different kinds of DNA isolation. The results of the analyses were compared with the respective information on food labelling.

MATERIAL AND METHODS

Samples. Samples of food from the Czech market were used in this study. A total of 57 food samples were tested, including 45 samples of animal origin and 12 samples of plant origin. Nine samples were soy products and were used as a positive control. The following food products were chosen as a positive control: soy flour, soy granulate, soy drink, soy bar, soy sauce, tofu chilli, tofu pate, soy sausage [plant-based product based on soya and wheat protein (water, soya protein 10.9%, rapeseed oil, wheat protein, modified starch, salt, aromas, spices, citrus fibre, carrageenan, extracts of spices)], pork sausage [pork sausages in inedible casings (pork 71%, water, table salt, soya protein, modified starch, pork collagen, stabilisers, aromas, antioxidants, preservatives)]. All of the samples were divided into three groups according to the information on the packaging: products containing soy (O), products containing no soy (N), and products containing traces of soy (S). The characteristics of the analysed samples and their grouping are shown in Table 1. After collection, the samples were marked with a code and stored at –18 °C (Mediline; Liebherr, Austria) until analysis or isolation. Sample preparation was dependent on the method used. All samples were mechanically homogenised (friction dish), and 200 mg of each sample was analysed by ELISA, by PCR using the CTAB method, and also by PCR using the GeneSpin kit.

ELISA kit (Veratox for Soy Allergen; Neogen, USA) was used for the quantitative analysis of soy residue in food products. This kit is a sandwich enzyme-linked immunosorbent assay, and sample preparation, extraction, and ELISA were performed according to the manufacturer's protocol. The kit detected soy proteins, the detection limit of ELISA is 0.96 ppm, and the measuring range was 2.5–25.0 mg kg^{-1} of soy flour. The absorbance was measured using a microwell reader with a 650 nm filter (Varioscan Flash; ThermoScientific, USA).

DNA isolation. The isolation of DNA was performed in two ways. The first involved the use of two different commercial kits – GeneSpin kit (Eurofins, Hungary; typical yields for GeneSpin are in the range of 0.1–10.0 μg DNA) and DNeasy Plant Pro (Qiagen, Germany; yields

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Table 1. Food samples and their classification into groups according to the information on packaging and the results of PCR, ELISA, and DNA concentrations

Sample No.	Product	Information on the label	PCR			ELISA (mg kg^{-1})	DNA* (ng µL^{-1})	A_{260}/A_{280}
			CTAB	GeneSpin	DNeasy Plant	(average ± SD)		
1	wheat flour	N	0	0	–	< 2.5	–	–
2	corn polenta	N	0	0	–	312.8 ± 191.9	69.12 ± 9.38	1.72
3	soy flour	C	1	1	1	892.5 ± 14.6	191.23 ± 5.62	1.44
4	soy granulate	C	1	1	1	906.8 ± 10.4	248.12 ± 7.83	1.67
5	soy drink	C	0	1	1	922.0 ± 4.52	44.33 ± 3.97	1.12
6	soy sauce	C	1	1	–	< 2.5	103.80 ± 0.00	1.13
7	tofu chilli	C	1	1	–	917.8 ± 0.56	212.55 ± 0.00	1.48
8	tofu pate	C	0	0	0	148.1 ± 107.5	72.90 ± 2.61	1.51
9	vegetable spread	S	0	0	–	< 2.5	66.97 ± 6.54	0.88
10	soy sausage	C	1	1	0	878.3 ± 61.1	90.34 ± 28.64	1.11
11	pork sausage	C	1	1	–	744.7 ± 21.6	497.93 ± 31.98	1.99
12	soy bar	C	1	1	–	866.6 ± 7.21	161.02 ± 8.32	1.59
13	vegetable spread	C	1	1	–	779.3 ± 20.85	568.95 ± 0.00	1.85
14	chicken mortadella	N	0	0	0	< 2.5	91.65 ± 13.55	1.88
15	salami	N	0	0	–	< 2.5	46.53 ± 0.91	2.09
16	salami	N	0	0	–	< 2.5	153.75 ± 7.08	1.81
17	chicken ham	N	0	0	0	< 2.5	111.31 ± 46.64	1.69
18	ham	N	0	0	0	< 2.5	88.76 ± 9.99	1.64
19	canned pork	N	0	0	0	< 2.5	219.27 ± 95.48	1.46
20	Brussels pate	N	0	0	0	< 2.5	565.18 ± 36.52	1.70
21	ham sausage	N	0	0	–	< 2.5	235.73 ± 28.19	1.60
22	chicken sausage	N	0	0	–	< 2.5	453.65 ± 12.37	1.77
23	liver pate	N	0	0	0	–	722.67 ± 284.59	1.94
24	ham mousse	N	0	0	0	< 2.5	269.17 ± 28.19	1.74
25	canned pork	S	0	0	0	< 2.5	132.97 ± 35.8	1.52
26	pate with pepper	C	1	1	–	< 2.5	289.65 ± 11.35	1.83
27	turkey ham	S	0	0	–	< 2.5	92.02 ± 6.59	1.71
28	canned pork	S	0	0	0	< 2.5	133.44 ± 3.98	1.57
29	ham salami	N	0	0	0	260.8 ± 95.6	147.54 ± 25.00	1.66
30	turkey salami	S	0	0	0	< 2.5	140.89 ± 11.17	1.52
31	canned beef	S	0	0	0	< 2.5	124.14 ± 22.85	1.50
32	chicken salami	S	0	0	0	< 2.5	122.28 ± 1.32	1.60
33	pork salami	S	0	0	0	< 2.5	105.08 ± 1.32	1.50
34	duck liver pate	N	0	0	–	< 2.5	1 019.22 ± 197.02	1.91
35	ham with eggs	N	0	0	0	< 2.5	62.74 ± 2.14	1.50
36	pate mix	S	0	0	0	< 2.5	903.18 ± 29.65	1.95
37	pate mix	S	0	0	–	< 2.5	236.84 ± 4.41	1.67
38	vegetable spread	N	0	0	0	< 2.5	24.69 ± 3.62	2.26
39	duck liver pate	C	1	1	0	< 2.5	256.14 ± 51.51	1.54
40	turkey salami	N	0	0	0	< 2.5	71.38 ± 12.24	1.51

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Table 1. To be continued

Sample No.	Product	Information on the label	PCR			ELISA (mg kg^{-1})	DNA* (ng µL^{-1})	A_{260}/A_{280}
			CTAB	GeneSpin	DNeasy Plant	(average ± SD)		
41	chicken pate	S	0	0	0	< 2.5	1 052.18 ± 33.72	1.99
42	pate	S	0	0	0	< 2.5	721.26 ± 133.75	2.03
43	ham mousse	S	0	0	0	165.1 ± 2.82	219.85 ± 5.54	1.60
44	vegetable spread	S	0	0	0	< 2.5	469.37 ± 18.29	1.58
45	fine salami	N	0	0	0	< 2.5	166.59 ± 16.74	1.99
46	ham salami	N	0	0	–	< 2.5	496.53 ± 30.405	2.12
47	chicken sausage	N	0	0	–	< 2.5	328.92 ± 112.02	1.94
48	sausage	N	0	0	–	< 2.5	216.06 ± 26.27	1.95
49	chicken ham	N	0	0	–	< 2.5	305.06 ± 74.63	1.99
50	sausage	N	0	0	–	< 2.5	324.90 ± 2.68	1.89
51	liver pate	N	0	0	0	< 2.5	1 126.38 ± 16.93	1.96
52	liver pate	N	0	0	0	< 2.5	1 246.02 ± 145.61	1.80
53	pate	S	0	0	–	< 2.5	607.07 ± 104.45	1.92
54	chicken ham mousse	N	0	0	0	< 2.5	414.16 ± 43.24	1.78
55	canned pork	N	0	0	–	< 2.5	151.03 ± 12.73	1.81
56	canned beef	N	0	0	0	< 2.5	145.27 ± 13.26	1.74
57	canned pork	N	0	0	–	< 2.5	83.64 ± 3.69	2.03

*Samples were isolated using cetyltrimethylammonium bromide (CTAB) method; C – contains soy; N – no soy; S – traces of soy; 0 – negative; 1 – positive; (–) – not measured; PCR – polymerase chain reaction; ELISA – enzyme-linked immunosorbent assay; DNA – deoxyribonucleic acid; A_{260}/A_{280} – ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and ribonucleic acid (RNA) [a ratio of ~1.8 is generally accepted as 'pure' for DNA (ThermoScientific 2009)]

are in the range of 3.0–30.0 µg DNA). The second involved the extraction of DNA using the CTAB method. This method was described in detail by Stefanova et al. (2013). After dilution in water, the quantification of DNA was performed by means of a NanoPhotometer N60 instrument (Implen, Germany).

Subsequently, samples were stored at –20 °C (Mediline; Leibherr, Austria) until PCR.

A blank (without samples) was included in each process of DNA isolation. In addition, the presence of a lectin-specific nucleotide sequence (soy internal gene) was detected in food samples by PCR. The primer sequence for the end-point PCR method was designed according to the already published (Stefanova et al. 2013) GMO3 (GCC CTC TAC TCC ACC CCC ATC C) and GMO4 (GCC CAT CTG CAA GCC TTT TTG TG) sequences (ELISABETH PHARMACON, Czech Republic). The expected length of the amplification product was 118 base pairs (bp) (Greiner et al. 2005).

PCR amplification. PCR was conducted in duplicates. Each reaction tube contained 1 µL DNA, 0.2 µmol L^{-1} of each primer (GMO3 and GMO4), 0.2 mmol L^{-1} of each deoxyribonucleotide triphosphate (dNTP), PCR buffer, 2.5 mmol L^{-1} $MgCl_2$ and 1U Taq Polymerase (Top-Bio, Czech Republic). The amplification had the following parameters: initial denaturation at 95 °C (10 min)$^{-1}$, 35 cycles consisting of denaturation at 95 °C (30 s)$^{-1}$, annealing at 64 °C (30 s)$^{-1}$ and an extension at 72 °C (1 min)$^{-1}$ and a final extension at 72 °C (3 min)$^{-1}$. The amplified fragments were analysed by electrophoresis in 1.7% agarose gel (PCR agarose; Top-Bio, Czech Republic). DNA in agarose gel was visualised by means of SYBR Green (Merc, USA) staining on a transilluminator (ELETTROFOR, Italy).

DNA concentration. The concentration of DNA was measured against a blank. The blank is a solution without dissolved DNA. A 2 µL aliquot of sample was used for each measurement on the NanoPhotometer N60 instrument (Implen, Germany). Each sample was measured twice, and the average value was calculated. The DNA concentration was measured in samples isolated using the CTAB method.

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Statistical analysis. Statistical analysis was performed using UNISAT for Excel 6.5. The results of the examined methods were tested statistically by McNemar's test to express the level of agreement among the results.

RESULTS AND DISCUSSION

The results of individual analyses for all samples are shown in Table 1. An example of the visualisation of a 1.7% agarose gel after the amplification of a specific DNA fragment of lectin is shown in Figure 1. It is obvious that the positive signal was in the form of a 118 bp DNA band.

Results after isolation via the CTAB method, GeneSpin kit, and DNeasy Plant kit. All of the samples were isolated via the CTAB method and GeneSpin kit, while 33 samples were extracted using DNeasy Plant kit. The results obtained after isolation by the CTAB method showed the presence of lectin in 10 of the 12 samples where the presence was declared on the package. Only in tofu pate and soy drink was the presence not confirmed. Analysis after isolation via GeneSpin kit confirmed the presence in 11 of the 12 samples which declared soy on the label. Only 6 samples marked as containing soy were isolated using DNeasy Plant kit, and only three samples were positive for soy. As with the previous methods, soy was not confirmed in tofu pate, which is a highly processed product, which could explain this finding.

Food products are mostly affected by thermal processes or interactions among different components of the matrix. Further, the DNeasy Plant kit did not confirm the presence of soy in liver pate or soy sausage. Mafra et al. (2008) showed that, with respect to highly processed products, the CTAB method provided the highest DNA yields in comparison with other commercial kits. We did not confirm these results because, in tofu pate, the soy has not been detected by either extraction method, and in soy drink, the CTAB method did not also detect soy but DNeasy Plant and GeneSpin did. None of the evaluated isolation methods (CTAB, GeneSpin kit and DNeasy Plant kit) found soy in any of the 30 samples that were marked as not containing soy – that is, the specific gene was not found in these samples. Fifteen samples were marked as containing traces of soy, but none of the detection methods confirmed the presence of soy traces.

Results of ELISA. A total of 56 samples were analysed using the ELISA method. The measured concentrations of soy protein are shown in Table 1. For statistical analysis, a concentration of soy protein below the range of quantification (2.5 mg kg^{-1}) was marked as 0 (without the presence of soy protein), and a concentration greater than 2.5 mg kg^{-1} was marked as 1 (the presence of soy protein). In the group of products marked as containing soy (12 samples), ELISA confirmed 9 samples to be positive. Three samples (soy sauce, duck liver pate, and pate with pepper) were below the concentration range detectable by ELISA. It is interesting that of the 29 analysed samples supposed to be free of soy, ELISA found 2 samples (ham salami and corn polenta) to contain soy protein. This could indicate cross-contamination during the measurement; however, this was ruled out by repeated measurements. We can consider

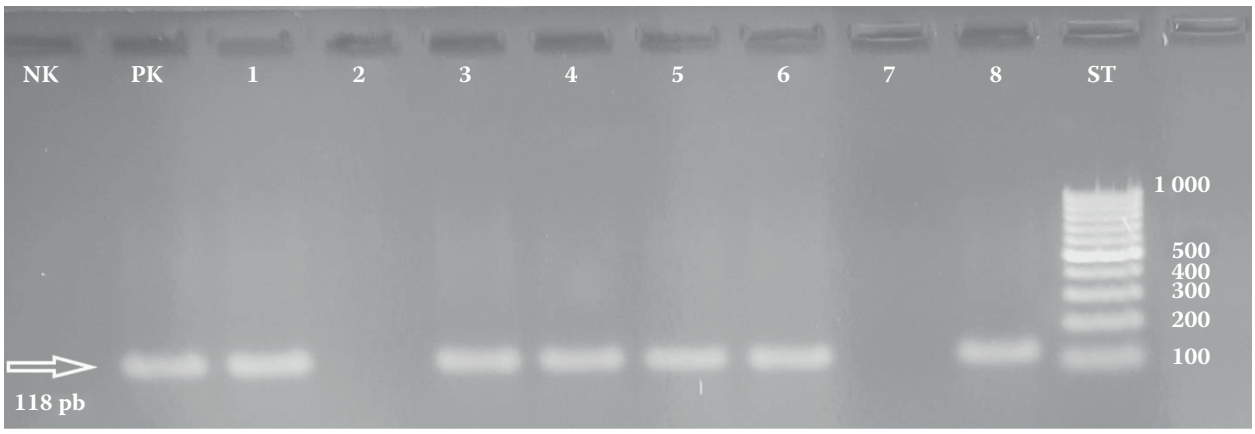

Figure 1. Agarose gel (1.7%) after the electrophoresis of PCR products (CTAB extraction method), with visualisation on a transilluminator after staining with SYBR Green

NK – negative control; PK – positive control (soy flour); 1 – soy granulate, 2 – soy drink, 3 – soy sauce, 4 – tofu chilli, 5 – soy bar, 6 – soy sausage, 7 – tofu pate, 8 – pork sausage samples; ST – length standard GeneRuler 100 base pairs (bp) deoxyribonucleic acid (DNA) Ladder; PCR – polymerase chain reaction; CTAB – cetyltrimethylammonium bromide

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that ham salami is a processed product where it can be difficult to detect soy using PCR. We can admit that soy was present in the ham salami, which was detected by ELISA, and the manufacturer then gives false information on the packaging. However, in the case of corn polenta, this does not seem likely to us. We can consider possible contamination during production in the company, which the producer did not indicate on the labelling. The producer declares on the labelling that the product contains 100% corn flour. On the other hand, Scharf et al. (2013) reported some false-positive results when using the ELISA method.

Comparison of the studied methods. ELISA and PCR using three types of DNA isolation were compared. The results of the statistical analysis are shown in Table 2. No significant differences were found between the methods, although for some samples, there was no agreement between the given methods with respect to soybean detection. Both PCR and ELISA methods demonstrated their valuable contribution to the determination of food allergens. The product group marked 'it may contain traces of soy' appears to be problematic. Soy was confirmed in only one of the 15 products thus marked, and only via ELISA.

There were, however, differences between the methods with respect to the demands for time and equipment. The least time-consuming was DNA isolation using DNeasy Plant kit, while DNA isolation via the CTAB method took the most time (Table 3). The most expensive of the tested isolation methods was GeneSpin kit, while the CTAB method was the least expensive.

Renčová and Tremlová (2009) measured soy in meat products in 2009 and found that soy protein was detected in 84% of samples without any declaration of soy on the packaging. In total, 131 meat products, salami and sausages from different producers were included in this study. This was performed before the implementation of Regulation No. 1169/2011 of the Parliament; three years after the regulation came into force, Brychta et al. (2014) found that only about 4% of samples without a declaration of soy on the label were tested positive for soy protein. Some other authors from the EU also detected soy in meat products and compared it with a declaration on the packaging. In Spain, Costa et al. (2016) tested meat products (25 samples of cooked ham and mortadella) when three samples were detected as not complying with labelling regulations as a result of undeclared soybean. Soares et al. (2014) analysed hamburgers, meatballs and nuggets and the results were all in compliance with the labelled soybean declared as an ingredient, but one sample of hamburger did not declare the amount of added soybean material which might suggest the fraudulent substitution of soybean proteins for meat. Jankovic et al. (2015) from Serbia found soy in 6% of the samples without declaration on the packaging. However, again with respect to meat products, Jankovic et al. (2015) found the presence of soybean protein in 29.6% of cases in which such protein was not indicated on the label, a situation posing a significant risk to the consumer. We detected soy in one sample where soy was not declared (ham salami), though the other three methods did not detect any soy in this sample. Our results, which are in compliance with some other authors (Brychta et al. 2014; Soares et al. 2014; Jankovic et al. 2015; Costa et al. 2016), show that the situation on the market is satisfactory because only sporadic samples were mislabelled. Only products declared as containing traces of soy appear to be problematic since the presence of soy has not been confirmed in any sample by any of the tested methods. As Sovová et al. (2020) wrote, if the manufacturer makes different kinds of products with different compositions, the products can contain traces of soybean due to cross-contamination, which cannot always be avoided. Soybean is an allergenic food, and the declaration of its even potential presence is obligatory in many countries [Regulation (EU) No. 1169/2011]. If not declared, the presence of soybean in the product can cause serious health problems in sensitive people (Sovová et al. 2020). As we have a look at the Rapid

Table 2. Results of McNemar's test – Comparison of the different methods (*P*-values)

Method	PCR/CTAB	PCR/GeneSpin	PCR/DNeasy Plant	ELISA
PCR/CTAB	–	0.9254	0.5338	0.4517
PCR/GeneSpin	0.9254	–	0.6783	0.5725
PCR/DNeasy Plant	0.5338	0.6783	–	0.9170
ELISA	0.4517	0.5725	0.9170	–

PCR – polymerase chain reaction; CTAB – cetyltrimethylammonium bromide; ELISA – enzyme-linked immunosorbent assay

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Table 3. Comparison of the time taken to conduct the given isolation methods and PCR (h:min)

Sample quantity	CTAB	DNeasy Plant	GeneSpin kit	PCR*
1 sample	4:20	0:22	1:15	4:05
50 samples	8:40	3:45	5:15	6:50

*Without the time required for the preparation of mastermix, the amplification of deoxyribonucleic acid (DNA), and electrophoretic separation; PCR – polymerase chain reaction; CTAB – cetyltrimethylammonium bromide

Alert System for Food and Feed (RASFF) portal, there is a total of 316 notifications from January 2017 to September 2020 relating to absent, incorrect, or incomplete labelling with respect to soy protein in food across the EU. Therefore, to protect consumers, it would be advisable for authorities responsible for food safety to continue to test all imported food and food products.

CONCLUSION

A total of four methods of determining soy in food were evaluated on 57 foods. According to our results, there is no significant difference between the tested methods, although some differences in highly technologically processed foods have been observed. After almost 10 years of the application of Regulation No. 1169/2011, 3% (2 out of 57) of products were found not to have information on the presence of soy on the packaging; however, these results were found only by the ELISA method, not by PCR.

A little unclear is the marking of the allergen traces, where the approaches to labelling may be confusing. The control of compliance with requirements by supervisory authorities is still necessary.

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