Semi-quantitative estimation of soya protein-based additives in meat products using real-time polymerase chain reaction

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Summary

A real-time polymerase chain reaction (PCR) detection system with a TaqMan probe for quantification of soya in meat products was developed and evaluated. The detection system targeted the *Le1* gene encoding for soya lectin and exhibited 100% inclusivity (9 *Glycine max* cultivars) and 100% exclusivity (22 plant samples) for the detection of soya. Analytical sensitivity of the assay was 5.75 pg DNA per reaction, which corresponds to approx. 2 haploid genome equivalents. Practical detection and quantification limits for three different soya protein-based additives (protein concentrate, protein isolate, okara), in liver paté as a matrix, were at a level of 0.1 g·kg^{-1}. The calibration lines for each series of model samples containing individual soya protein-based additives were statistically identical in the range from limit of quantification to 100 g·kg^{-1}, which means that the same calibration line could be used for all soya protein-based additives. The developed real-time PCR facilitated semi-quantitative estimation of soya protein-based additives in meat products, which may be useful for the control of labelling of meat products.

Keywords

soya; protein; meat; polymerase chain reaction

Soya beans (*Glycine max*) belong to important legumes and oil seeds. The defatted soya meal, which is in fact a by-product of soya vegetable oil production, is a good and cheap source of proteins that are widely used as a less expensive substitute for meat proteins in many food products. For a technologically effective use in the meat industry, soya protein-based additives are produced from soya meal. This is done basically by removing the water-soluble saccharides and, for a certain type of products, by extraction of other non-protein components. Depending on the way of processing of the soya meal, soya protein-based additives of different protein contents in the range between approx. 650 g·kg^{-1} and 950 g·kg^{-1} are produced. The products with the protein contents of approx. 650 g·kg^{-1} are usually designated protein concentrates, while products with the protein contents above 900 g·kg^{-1} are designated protein isolates, the latter being intended for use in pharmaceutical applications. Another protein-containing by-product from processing soya beans is okara, which is soya pulp consisting of insoluble components remaining in the production of soya milk. Dried okara, which can be used as an additive in production of certain food products, contains approx. 240 g·kg^{-1} proteins [1, 2].

Because soya is an allergen, its presence in food products has to be declared on the label [3, 4]. In this case, an analytical method should be able to quantify soya in the range from 1 g·kg^{-1} down to less than 0.01 g·kg^{-1} [3, 5]. However, content of soya in food products is of interest also from a point of view of adulteration, since a variety of processed meat products contain soya protein-based additives as a substitute for a part of

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the meat. If such substitution is not properly declared on the label, it may be classified as fraud. In this case, an analytical method should be able to quantify soya in the range from 10 $g \cdot kg^{-1}$ to 100 $g \cdot kg^{-1}$ [1].

For the purpose of controlling proper food labelling, several methods for detection of soya in food products were developed. These are based either on direct detection of proteins by enzyme-linked immunosorbent assay (ELISA) [6–8] or are based on indirect detection of soya DNA by polymerase chain reaction (PCR) [9–13]. Alternative methods such as immunohistochemical technique [14] or near-infrared spectroscopy [15] are also available. However, the analytical methods so far available were originally developed with the main aim of detection of allergens as sensitively as possible, and the potential of the analytical methods to quantify soya in the content range relevant for food authentication was not evaluated. The situation at DNA-based detection of soya in food may be further complicated if different soya protein-based additives (e.g. protein concentrates or protein isolates) contain different levels of DNA. In this case, DNA-based quantification of soya would not be feasible without a priori knowledge of the type of additive present in the food product. Although a preliminary information was encouraging, saying that various soya protein concentrates contain identical DNA levels (E. Jaccaud, personal communication), this fact needed to be confirmed. The evaluation was the subject of the present study, involving three different types of soya protein-based food additives. In this study, a modified real-time PCR method was developed, targeting an established soya-specific marker gene *Le1*, and its potential for quantification of soya in meat products was assessed.

MATERIALS AND METHODS

Samples

Soya cultivars were obtained from Gene Bank of the Slovak Republic, Research Institute of Plant Production, Piešťany, Slovakia. Soya protein concentrates Arcon S (Archer Daniels Midland, declared protein content 720 $g \cdot kg^{-1}$) and Pro-Fam 974 (Archer Daniels Midland, declared protein content 900 $g \cdot kg^{-1}$) were obtained from Amitco (Brno, Czech Republic). Dried okara (declared protein content 240 $g \cdot kg^{-1}$) was obtained from Sojaprodukt (Drietoma, Slovakia). Liver paté Májka (Hamé, Kunovice, Czech Republic) was obtained from the market in Brno, Czech Republic; absence of soya in the matrix was checked by PCR according to Meyer et al. [9]. Weighed amounts of a soya protein-based additive were thoroughly mixed using mortar and pestle during 10 min with a calculated amount of liver paté in batches of 5 g, and serial dilution with the matrix was applied to obtain model samples containing from 100 $g \cdot kg^{-1}$ to 0.1 $g \cdot kg^{-1}$ of individual soya protein-based additives. Seeds of different soya cultivars were obtained from a specialized shop in Bratislava, Slovakia. Further plant and meat samples were obtained from the markets in Bratislava, Slovakia.

DNA extraction

DNA was extracted from homogenized samples (200 mg) by chaotropic solid phase extraction (SPE) using NucleoSpin Food Kit (Macherey-Nagel, Düren, Germany) in accordance with the instructions for use attached to the kit. All extractions included a blank for the control of reagents and possible contamination during the extraction procedure. Amplifiability of the extracted DNA was checked by universal eukaryotic real-time PCR [16]. DNA concentration was determined by fluorimetry using Quant-iT PicoGreen kit (Invitrogen Molecular Probes, Eugene, Oregon, USA). DNA for the determination of inclusivity, DNA-based limit of detecion (*LOD*) and DNA-based limit of quantification (*LOQ*), was isolated from leaves of *G. max* by chaotropic SPE using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

Polymerase chain reaction

Real-time PCR assays were performed in individual reaction volumes of 20 μl. Each reaction tube contained 2 μl of DNA solution (50–100 ng), 1.5 U Cheetah Hot Start Taq polymerase (Biotium, Hayward, California, USA), reaction buffer

Tab. 1. Primers and probe used in the study.

Designation	Sequence (5'-3')
BLe2F	CCA GCT TCG CCG CTT CCT TC
BLe2R	GAA GGC AAG CCC ATC TGC AAG CC
BLe2P	FAM-CTT CAC CTT CTA TGC CCC TGA CAC-TAMRA

supplied with the DNA polymerase, 2.5 mmol·l^{-1} $MgCl_2$ and 200 μmol·l^{-1} dNTP mixture (Applied Biosystems, Foster City, California, USA). Primers BLe2F and BLe2R were used at concentrations of 300 nmol·l^{-1} and the probe BLe2P was used at a concentration of 200 nmol·l^{-1}. Oligonucleotides, sequences of which are given in Tab. 1, were synthesized by Eurofins MWG Operon (Ebersberg, Germany). Amplification was carried out in a real-time PCR cycler model 7900 (Applied Biosystems) using a temperature programme consisting of initial denaturation at 95 °C for 5 min, followed by 50 cycles of denaturation at 95 °C and annealing with polymerization at 65 °C for 60 s. Data on fluorescence in optical channel for FAM (6-carboxyfluorescein) were collected and amplification curves were processed using the internal software of the instrument. In case of exclusivity testing, the reaction mixture contained an internal amplification control (IAC) system (Applied Biosystems, cat. no. 4308323), which was monitored in optical channel for VIC (4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein). In qualitative analysis, an increasing amplification curve with quantification cycle lower than 36 was taken as the positive result, in order to avoid false positivity originating in amplification-independent fluorescence increase. All analyses were done in triplicate, if not otherwise stated.

Statistics

Results were statistically processed, including linear regression and analysis of covariance (ANCOVA), using Prism 5 Software (GraphPad, San Diego, California, USA).

RESULTS AND DISCUSSION

An analytical method providing data on the contents of soya in processed meat products would be useful for control of correctness of food labelling. The previously published real-time PCR-based methods demonstrated a potential for such quantification [11, 13]. In this study, we aimed at improvement of the quantification potential of the method, and at testing its practical quantification potential, in a model matrix with defined additions of different soya protein-based additives.

A new set of PCR primers BLe2F, BLe2R and a 5'-hydrolase (TaqMan) probe BLe2P was designed on the sequence of an established soya-specific marker gene *Le1* (GenBank accession number K00821; Tab. 1). The system was designed to have as low as possible amplicon length, which was 74 bp. The real-time PCR with these primers and the probe systems exhibited 100% inclusivity when tested with 9 soya cultivars (Tab. 2) and 100% exclusivity when tested on 22 different plant samples (Tab. 3).

Tab. 2. Inclusivity of real-time PCR.

Soya cultivar	Detection result
Belmont	+
Cardiff	+
Enterprise	+
Kanakawa Wase	+
Korada	+
OAC Erin	+
OAC Vision	+
Primus	+
Quito	+

(+) – positive PCR result.

Tab. 3. Exclusivity of real-time PCR.

Species / genus	Cultivar / specificiation	Detection result
Phaseolus vulgaris	Katka	–
Phaseolus vulgaris	Maxidor	–
Phaseolus vulgaris	White butter beans	–
Pisum sativum	Gloriosa	–
Pisum sativum	Progress	–
Pisum sativum	Zázrak z Kelvedonu	–
Almond	kernels	–
Brazil nuts	kernels	–
Cashew	nuts	–
Chickpea	seeds	–
Hazelnut	kernels	–
Macadamia	kernels	–
Maize	flour	–
Oat	flakes	–
Peanut	seeds	–
Pecan nuts	kernels	–
Pistachio	kernels	–
Rice	kernels	–
Rye	flour	–
Sunflower	seeds	–
Walnut	kernels	–
Wheat	flour	–

(–) – negative PCR result.

Analytical sensitivity of the assay was determined on the basis of analysis of a series of decimally diluted solutions of DNA isolated from *G. max cv* Korada leaves. This parameter was found to be equal to 5.75 pg per reaction (Fig. 1), which is equivalent to approx. 2 haploid genomes

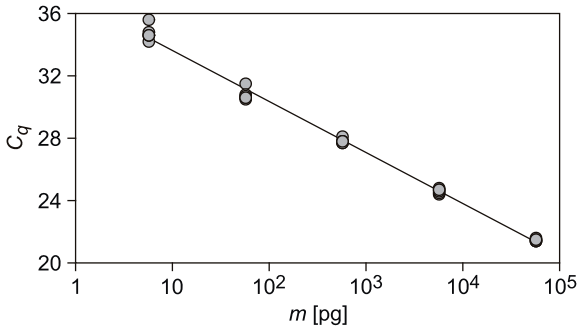

Fig. 1. Calibration line for determination of analytical sensitivity of the assay.

Analysis was done in 6 replicates. C_q – quantification cycle, m – weight of DNA (per reaction aliquot).

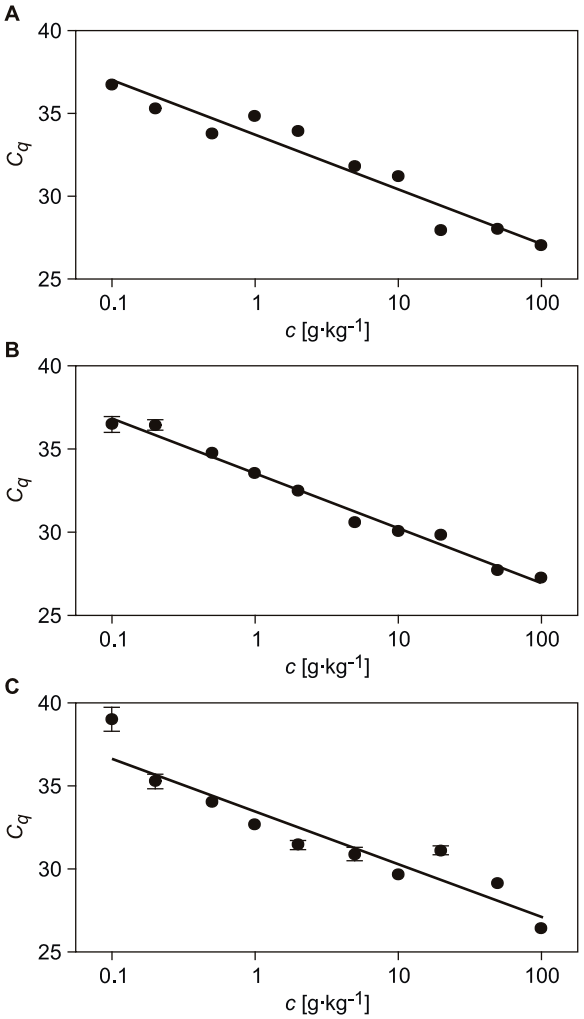

Fig. 2. Calibration lines of real-time PCR with series of spiked model liver paté samples.

A – paté spiked with Arcon S, B – paté spiked with Pro-Fam, C – paté spiked with Okara.
Points represent means of three replicate analyses with bars representing standard deviation, if great enough to be depicted. C_q – quantification cycle, c - content of soya protein-based additive in paté.

per reaction, given the genome size of *A. hypogaea* is approx. 2.96 pg [17]. A calibration line used to calculate *LOD* and *LOQ* is presented in Fig. 1. This analytical parameter is comparable with those reported previously for other soya-specific real-time PCR methods [11–13].

Parameters of practical analytical applicability were determined for the real-time PCR based on the results of analysis of a series of model samples containing a defined addition of soya protein-based additive. Liver paté was used as a basic matrix for the preparation of model samples, because it was found to facilitate very good homogenity with added soya protein additives in our preliminary experiments. Three different types of soya protein-based additives, namely, soya protein concentrate, soya protein isolate and okara, were used. Results shown in Fig. 2 and Tab. 4 illustrate the performance of real-time PCR. The data were analysed by ANCOVA and all three calibration lines were found to be statistically identical, with identical slopes (p = 0.8312) and identical intercepts (p = 0.7622). This result meant that a pooled calibration line could be constructed from all the data, with a pooled slope k = –3.23875 and a pooled Y intercept q = 30.349.

The precision of the analytical method could be improved by increasing the number of replicate analyses to 6 or 12, with appropriate statistical treatment. This would improve the linearity of calibration lines, reflected by correlation coefficients getting closer to 1. However, certain difficulties should be always anticipated at attempts to precisely quantify food components by real-time PCR, even in homogenous samples. An important intrinsic factor in this respect is the small slope of the calibration line of real-time PCR, on a logarithmic concentration scale [18, 19]. Measures should be applied to provide a maximum of achievable precision and accuracy, regarding sampling, sample preparation and number of replicates when the method is implemented to routine food testing [20, 21].

Since statistically identical calibration lines were obtained for series of model paté samples spiked with different soya protein-based additives, this suggests that the real-time PCR-based method can facilitate semi-quantitative estimation of soya in meat products without a priori knowledge of the type of the additive. In practical case, a series of spiked samples should be prepared for calibration purposes and, using the obtained calibration line, unknown samples can be quantified. The spiked samples should be prepared using a matrix as similar as possible to that of the analysed samples and, for spiking, a probable type of soya-based protein

Tab. 4. Parameters of calibration lines for the real-time PCR with three series of model samples containing different types of soya protein-based additives.

Soya protein-based additive	Slope	Y-intercept	R^2
Arcon S	–3.272	30.43	0.9303
Pro-Fam	–3.287	30.28	0.9776
Okara	–3.149	30.33	0.8696

additive should be used, based on the information about the analysed food product or other information from the food industry.

CONCLUSION

A real-time PCR detection system with a TaqMan probe for quantification of soya was developed and found to have good parameters of 100% inclusivity, 100% exclusivity and analytical sensitivity of 5.75 pg DNA per reaction, which corresponds to approx. 2 haploid genome equivalents. When this real-time PCR was applied to DNA isolated from three series of liver paté samples spiked with three different soya protein additives, statistically identical calibration lines were obtained. This means that semi-quantitative estimation of soya protein-based additives in meat products can be obtained by using this real-time PCR-based method, when calibrated by a series of spiked samples based on roughly similar soya protein-based additives.

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REFERENCES

1. Enders, J. G.: Soy protein products – Characteristics, nutritional aspects, and utilization. Revised and expanded edition. Champaign : AOCS Press, 2001. ISBN 1-893997-27-8.
2. Friedman, M. – Brandon, D. L.: Nutritional and health benefits of soy proteins. Journal of Agricultural and Food Chemistry, *49*, 2001, pp. 1069–1086. DOI: 10.1021/jf0009246.
3. Bush, R. K. – Hefle, S. L.: Food allergens. Critical Reviews in Food Science and Nutrition, *36,* 1996, Suppl. 1, pp. S119–S163. DOI: 10.1080/10408399609527762.
4. Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. Official Journal of European Communities, *L308*, 2003, pp. 15–18.
5. Pedersen, M. H. – Holzhauser, T. – Bisson, C. – Conti, A. – Jensen, L. B. – Skov, P. S. – Bindslev-Jensen, C. – Brinch, D. S. – Poulsen, L. K.: Soybean allergen detection methods – A comparison study. Molecular Nutrition and Food Research, *52*, 2008, pp. 1486–1496. DOI: 10.1002/mnfr.200700394.
6. Koppelman, S. J. – Lakemond, C. M. M. – Vlooswijk, R. – Hefle, S. L.: Detection of soy proteins in processed foods: Literature overview and new experimental work. Journal of AOAC International, *87*, 2004, pp. 1398–1407.
7. Asensio, L. – González, I. – García, T. – Martín, R.: Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA). Food Control, *19*, 2008, pp. 1–8. DOI: 10.1016/j.foodcont.2007.02.010.
8. Cucu, T. – Devreese, B. – Kerkaert, B. – Rogge, M. – Vercruysse, L. – De Meulenaer, B.: ELISA-based detection of soybean proteins: A comparative study using antibodies against modified and native proteins. Food Analytical Methods, *5*, 2012, pp. 1121–1130. DOI: 10.1007/s12161-011-9341-y.
9. Meyer, R. – Chardonnens, F. – Hübner, P. – Lüthy, J.: Polymerase chain reaction in the quality assurance and safety of food: detection of soya in processed meat products. Zeitschrift für Lebensmittel-Untersuchung und–Forschung, *203*, 1996, pp. 339–344. DOI: 10.1007/BF01231072.
10. Gryson, N. – Messens, K. – Dewettinck, K.: PCR detection of soy ingredients in bread. European Food Research and Technology, *227*, 2007, pp. 345–351. DOI: 10.1007/s00217-007-0727-4.
11. Soares, S. – Mafra, I. – Amaral, J. S. – Oliveira, M. B. P. P.: A PCR assay to detect trace amounts of soybean in meat sausages. International Journal of Food Science and Technology, *45*, 2010, pp. 2581–2588. DOI: 10.1111/j.1365-2621.2010.02421.x.
12. Espiñeira, M. – Herrero, B. – Vieites, J. M. – Santaclara, F. J.: Validation of end-point and real-time PCR methods for the rapid detection of soy allergen in processed products. Food Additives and Contaminants, *27*, 2010, pp. 426–432. DOI: 10.1080/19440040903493777.
13. Köppel, R. – Dvorak, V. – Zimmerli, F. – Breitenmoser, A. – Eugster, A. – Waiblinger, H. U.: Two tetraplex real-time PCR for detection and quantification of DNA from eight allergens in food. European Food Research and Technology, *230*, 2010, pp. 367–374. DOI: 10.1007/s00217-009-1164-3.
14. Pospiech, M. – Tremlová, B. – Renčová, E. – Randulová, Z. – Řezáčová Lukášková, Z. – Pokorná, J.: Comparison of the results of the ELISA, histochemical and immunohistochemical detection of soya proteins in meat products. Czech Journal of Food Sciences, *29*, 2011, pp. 471–479.
15. Li, H. – Wang, J. – Li, J. – Yang, H. – Qin, Y.: Quantitative determination of soybean meal content in compound feeds: Comparison of near-infrared

spectroscopy and real-time PCR. Analytical and Bioanalytical Chemistry, *389*, 2007, pp. 2313–2322. DOI: 10.1007/s00216-007-1624-1.
16. Brežná, B. – Hudecová, L. – Kuchta, T.: Detection of pea in food by real-time polymerase chain reaction (PCR). European Food Research and Technology, *222*, 2006, pp. 600–603. DOI: 10.1007/s00217-005-0168-x.
17. Temsch, E. M. – Greilhuber, J.: Genome size variation in *Arachis hypogaea* and *A. monticola* re-evaluated. Genome, *43*, 2000, pp. 449–451. DOI: 10.1139/g99-130.
18. Janská, V. – Piknová, L. – Kuchta, T.: Semi-quantitative estimation of the walnut content in fillings of bakery products using real-time polymerase chain reaction with internal standard material. European Food Research and Technology, *235*, 2012, pp. 1033–1038. DOI: 10.1007/s0217-012-1832-6.
19. Janská, V. – Piknová, L. – Kuchta, T.: Quantification of hazelnut content in fillings of cakes and wafers using real-time polymerase chain reaction. Food Analytical Methods, *6*, 2013, pp. 1492–1495. DOI: 10.1007/s12161-013-9663-z.
20. Laube, I. – Zagon, J. – Broll, H.: Quantitative determination of commercially relevant species in foods by real-time PCR. International Journal of Food Science and Technology, *42*, 2007, pp. 336–341. DOI: 10.1111/j.1365-2621.2006.01249.x.
21. Mazzara, M. – Savini, C. – Charles Delobel, C. – Broll, H. – Damant, A. – Paoletti, C. – Van den Eede, G.: Definition of minimum performance requirements for analytical methods of GMO testing. Ispra : Joint Research Centre, 2008. DOI: 10.2788/65827.

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