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Detecting soybean and milk in dairy and soy products with post-PCR high resolution melting assays

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Abstract: We have developed and validated post-PCR (polymerase chain reaction) high resolution melting (HRM) based assays that allow identifying the presence of two major allergenic foods – soybean and bovine milk – in food products. A new set of primers for PCR was developed for detection of the gene encoding the soybean protein lectin. The assay was validated using reference samples and used for analysis of artificially prepared mixed samples of dairy and soy products of different matrices, as well as of real products available in the market (spray creams). The limits of detection (LODs) of the soybean (8 copies) and bovine milk (4 copies) assays were lower compared to LODs of other previously published PCR-based assays. The analysis of several commercial spray creams revealed an undeclared presence of soybean in one of the samples. The newly developed HRM assays are precise and robust alternatives for the control of food composition and falsification by competent authorities.

Keywords: food safety; food quality; food analysis; allergen; *Glycine max* L.

The quality and safety of foods are of utmost importance and are increasingly followed by both consumers and state authorities. The food on consumers' tables may come from very different sources and it is thus necessary to confirm the source of the food products (Lockley & Bardsley 2000). Occasionally, food producers replace more expensive ingredients with cheaper alternatives to increase the profit; it is therefore necessary to investigate the true product composition to avoid deception and fraud. Moreover, with the increasing prevalence of food allergies, the detection of trace amounts of food constituents is crucial to protect public health (Nwaru et al. 2014). A dependable system of food controls based on sensitive and robust analytical methods is thus necessary for food constituent or contaminant detection and species identification.

Dairy products and their substitutes, such as soybean, are a prime example of this necessity. Both milk and soybean are major allergenic foods (Foucard & Malmheden Yman 1999; Nwaru et al. 2014). In Europe, about 6.0% and 2.5% of the population declare a self-reported lifetime prevalence of allergy to cow's milk and soybean, respectively (Nwaru et al. 2014). Soybean has even been shown to cause life-threatening reactions in sensitive patients (Foucard & Malmheden Yman 1999). Undeclared substitution of soy alternatives for milk protein is a fraudulent practice, and soy products need to be checked since people allergic to milk proteins use it as an alternative to dairy. The analysis and proper labelling of milk and soy products is therefore of great importance.

Several approaches have been used for milk and soybean detection in food products. These methods include immunological (Song et al. 2011; Ren et al. 2014), electrophoretic (Wu et al. 2014; Ibáñez et al. 2016) and chromatographic (Jablonski et al. 2014; Rani et al. 2015) techniques which detect specific differences in protein or fatty acid composition of the products.

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The protein-based methods are however unsuitable for the analysis of heat-treated materials as proteins get denatured, whereas chromatographic methods are expensive and quite laborious for routine applications (Lockley & Bardsley 2000). Therefore, using DNA-based methods, namely polymerase chain reaction (PCR) and its variants like quantitative real-time PCR (qPCR), represents a suitable alternative in many areas of food analysis due to simplicity, reliability, cost efficiency, and stability of DNA during food processing (Lockley & Bardsley 2000). (q)PCR has been used to analyse various food constituents and several studies have also investigated detection of soybean in dairy products (Phipps et al. 2003) and *vice versa* (Mayer et al. 2012). Another option is to use post-PCR techniques, including high resolution melting (HRM). HRM consists in measuring the rate by which a double-stranded DNA dissociates (or melts) to single-stranded DNA with rising temperature using an intercalating fluorescent dye. The melting behaviour is distinctive of the DNA segment. In general, HRM technique allows accurate and sensitive quantitation of adulterants in commercial food products both plant- and animal-derived.

In this study, we developed post-PCR HRM assays for detection of bovine DNA in soy products and *vice versa*. A new set of primers for PCR was developed for detection of the gene encoding the soybean protein lectin. The assays were optimised and validated, and used for the analysis of both artificially prepared samples of dairy and soy products of different matrices and real products available in the market.

MATERIAL AND METHODS

Samples and DNA extraction. All soy and dairy samples used in this study have been obtained from Milcom Corp. (Czech Republic) – a Czech research and production company in the field of dairy industry. Organic quality soybeans and pure pasteurised organic bovine milk were used as reference samples to validate the assay and as positive control samples.

To verify the applicability of the assay to milk and soy DNA isolated from samples with different matrices, fifteen composite samples were deliberately prepared by mixing a range of soy and bovine milk products in various proportions (samples M1–M15, Table 1). Eight samples were prepared by adding a soy

Table 1. Results of HRM analysis of the presence of bovine (COI) and soybean (Lec118) DNA in 15 samples artificially prepared by mixing different dairy and soybean products in varying proportions

Sample	Products	Soybean product content (% w/w)	Melting point COI (°C)	Melting point Lec118 (°C)
Bovine milk		0	75.8 ± 0.1	ND
Soybean		100	ND	81.1 ± 0.1
M1	bovine milk yogurt, freeze-dried soy bacterial culture	1	76.2 ± 0.1	81.1 ± 0.1
M2	butter spread, soy flour	3	76.0 ± 0.1	81.1 ± 0.0
M3	dairy nutritional supplement, soy flour	6	76.0 ± 0.1	81.1 ± 0.0
M4	bovine milk quark, tofu	9	76.1 ± 0.0	81.2 ± 0.1
M5	bovine milk yogurt, dried soy milk	9	76.0 ± 0.1	81.1 ± 0.0
M6	bovine milk quark, dried soy milk	10	76.0 ± 0.0	81.0 ± 0.1
M7	semi-skimmed bovine milk, dried soy milk	12	75.5 ± 0.1	80.4 ± 0.1
M8	yogurt from semi-skimmed bovine and soy milk	24	76.1 ± 0.0	81.1 ± 0.1
M9	soy cream, dairy cream	70	75.8 ± 0.4	81.0 ± 0.0
M10	soy yogurt, semi-skimmed bovine milk	75	75.6 ± 0.1	80.7 ± 0.1
M11	dried soy milk, dried skimmed bovine milk	75	75.5 ± 0.1	80.6 ± 0.0
M12	tofu spread, semi-skimmed bovine milk	86	76.1 ± 0.1	81.2 ± 0.1
M13	tofu spread, bovine milk yogurt	89	75.9 ± 0.3	81.3 ± 0.0
M14	yogurt from soy milk and bovine milk culture	91	75.8 ± 0.1	81.0 ± 0.0
M15	soy yogurt, skimmed bovine milk	97	75.6 ± 0.1	80.8 ± 0.1

Data are mean ± SD (n = 3); ND – not detected

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Table 2. Results of HRM analysis of the presence of bovine (COI) and soybean (Lec118) DNA in ten commercial spray cream samples

Sample	Containing vegetable fats/ containing soybean/ potential soybean presence declared	Melting point COI (°C)	Melting point Lec118 (°C)
Bovine milk	–	75.8 ± 0.1	ND
Soybean	–	ND	81.2 ± 0.1
C1	No/No/No	76.2 ± 0.1	80.9 ± 0.0
C2	No/No/No	76.3 ± 0.0	ND
C3	No/No/No	76.3 ± 0.1	ND
C4	No/No/No	76.2 ± 0.1	ND
C5	No/No/No	76.2 ± 0.1	ND
C6	No/No/No	76.3 ± 0.1	ND
C7	No/No/Yes	75.7 ± 0.1	80.6 ± 0.2
C8	Yes/No/No	76.3 ± 0.1	ND
C9	Yes/No/No	76.2 ± 0.1	ND
C10	Yes/No/No	75.9 ± 0.1	ND

Data are the mean ± SD (n = 3); HRM – high resolution melting; ND – not detected

product to a dairy product such as yoghurt, cream or spread (M1–M8) and seven samples were prepared by adding a dairy product to various soy products such as tofu, soy milk or flour (M9–M15).

Furthermore, ten different commercial spray creams (samples C1–C10, Table 2) were purchased at local supermarkets and tested for the presence of bovine milk and soybean. The samples included seven dairy-only spray creams with no added vegetable fat (C1–C7) and three dairy spray creams enriched with vegetable fats (C8–C10).

The DNA from all of the above-mentioned samples was isolated using the cetyltrimethylammonium bromide (CTAB) method in accordance with the ISO 21571:2005 standard and dissolved in 60 µL of Tris-EDTA (TE) buffer. The concentration and purity of the DNA were determined using UV spectrophotometry (NanoPhotometer P300; Implen, Germany) and by gel electrophoresis. In all cases, good quality DNA of sufficient purity was obtained.

HRM assay. All primers used for the post-PCR HRM (High Resolution Melting) analyses are listed in Table 3. For detection of bovine milk, a primer pair (COI-F and COI-R) previously designed by Feligini *et al.* (2005) for amplification of the bovine-specific region of the cytochrome oxidase subunit I (COI) gene was used. For detection of soybean, a new primer pair (Lec118-F and Lec118-R) was designed to amplify a 118-bp fragment of the soy lectin gene based on the DNA sequence from the NCBI database (accession code K00821.1) using the freely accessible software Primer3Plus. The specificity of both primer pairs was verified *in silico* using NCBI BLAST tool and *in vitro* using several plant (rice, wheat, maize, flax, mustard, papaya, canola, garlic, onion, cranberry, hops) and animal (sheep, goat, chicken, turkey) DNA.

Table 3. Assay oligonucleotides used for HRM analyses for detection of bovine (COI) and soybean (Lec118) DNA

Assay	Oligo	Nucleotide sequence (5´–3´)	Length (bp)	Source
COI	Forward (COI-F)	GAACTCTGCTCGGAGACGAC	20	Feligini et al. (2005)
	Reverse (COI-R)	AGCACCAATTATTAGGGGAAC	21	
Lec118	Forward (Lec118-F)	GTGACCTCCTCGGGAAAGTT	20	This research
	Reverse (Lec118-R)	CGGTTTCTTTGTCCCAAATG	20	

Bp – base pairs

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Amplification and melt curve analysis were performed in StepOnePlus real-time PCR systems (Applied Biosystems, USA); data were analysed with the HRM Software 3.0.1 (Applied Biosystems, USA). The reaction of 25 µL had the following reaction mixture components (final concentrations): 1 × MeltDoctor™ (Applied Biosystems, USA) and 300 nM of the forward and reverse primers. The remainder of the reaction mixture volume consisted of 5 µL of the DNA solution and 3.8 µL of nuclease-free water (NFW; Sigma, Germany). DNA concentration did not exceed 20 ng μL^{-1}. The amplification temperature profile was as follows: Initial denaturation at 95 °C for 600 s, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 60 s with fluorescence acquisition after each 60 °C step. The melt curve analysis was performed from 60 °C to 95 °C (ramp rate of 0.1 °C s^{-1}).

To determine the reference melting temperatures and validate the assay, the reference samples (bovine milk and soybean) were analysed in ten replicates and three independent runs. Moreover, limits of detection (LODs) of the two post-PCR HRM assays (COI and lectin) were determined using the reference DNA samples which were serially diluted using NFW (2.69×10^{-5}–0.55 ng for bovine DNA and 1.25×10^{-3}–1.6 ng for soybean DNA). Each reaction was performed in ten replicates; the lowest DNA concentration resulting in a positive and specific response (melt curve) in all ten replicates was set as the LOD.

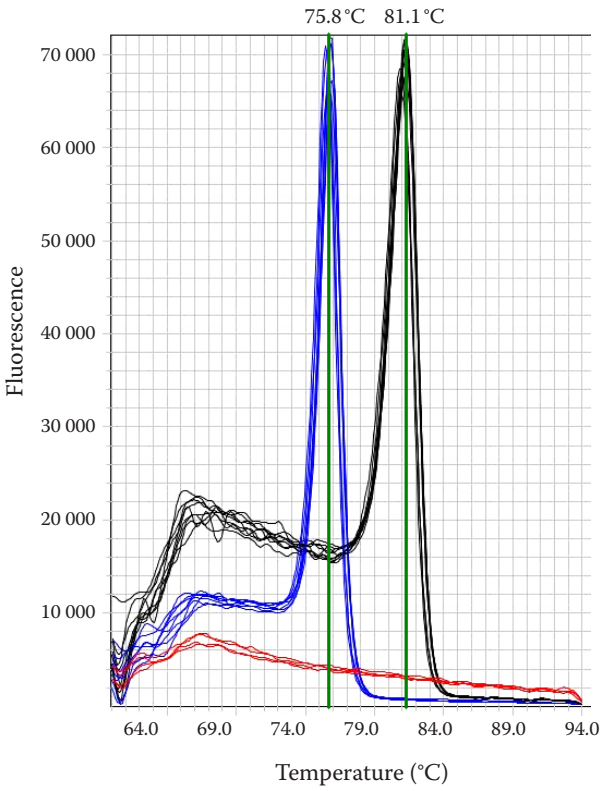

Figure 1. Representative aligned melting curves of HRM assays of the presence of bovine (COI) and soybean (Lec118) DNA in samples isolated from bovine milk and soybeans, respectively (n = 10)

Black curves – COI; blue curves – Lec118; red curves – negative control samples

RESULTS AND DISCUSSION

The Lec118 primers for detection of the soy lectin gene were newly designed in this study. The BLAST (Basic Local Alignment Search Tool) sequence similarity search confirmed the primer specificity (data not shown). Moreover, the specificity of the primer pairs was verified using several animal and plant DNAs; the results were negative for all samples (data not shown). Both assays were validated using the DNA templates extracted from bovine milk and soybean, and two distinct melting profiles were obtained (Figure 1). The melting points for COI and Lec118 amplicons reflecting the presence of the bovine and soy DNA in the reference samples were 75.8 ± 0.1 °C and 81.1 ± 0.1 °C [mean ± standard deviation (SD), n = 30] with the coefficient of variation of 0.16% and 0.12%, respectively. Practical LODs for the assays were determined to be 9.1 pg of soybean DNA (i.e. 8 copies) and 0.4 pg of bovine DNA (i.e. 4 copies). Sensitivity (defined by LOD) of the Lec118 [25–50 pg in end-point PCR (Phipps et al. 2002; Phipps et al. 2003; Rott et al. 2004; Espiñeira et al. 2010); 9.8–20 pg in qPCR (Berdal & Holst-Jensen 2001; Espiñeira et al. 2010; Costa et al. 2017)] and COI assays [1–5 pg in PCR (Rea et al. 2001; Mafra et al. 2004); 0.5–200 pg in qPCR (Walker et al. 2003; López-Calleja et al. 2005; López-Calleja et al. 2007; Dalmasso et al. 2011; Mayer et al. 2012); 20–200 pg in HRM (Sakaridis et al. 2013a; 2013b)] was higher than that of other reported assays detecting soybean/bovine milk in food products. The designed HRM assay is thus suitable for detection of soy additives in dairy milk products and *vice versa* even at very low concentrations.

The applicability for detection of soybean and bovine milk in samples of different matrices (fat content, heat or microbial processing, etc.) was verified in fifteen mixed samples (Table 1). The results of the COI assay showed the presence of a signal specific to bovine milk DNA in all tested samples with the exception of the soybean reference sample. The same was observed

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in the Lec118 assay when soybean was detected in all tested samples with the exception of the bovine milk reference sample. Therefore, both bovine milk and soy DNA were detected in the tested samples regardless of the used concentration of the dairy/soy ingredient and its nature.

Furthermore, the assays were used to analyse real commercial samples – ten spray creams purchased in local supermarkets (Table 2). To lower costs, spray creams are often enriched with various vegetable fats that can contain traces of soybean. The soy phospholipid lecithin can also be added as an emulsifier to increase the stability of the cream. Moreover, if the manufacturer makes different kinds of products with different composition, the products can contain traces of soybean due to cross-contamination which cannot always be avoided. Therefore, since soybean is a powerful allergenic food, the declaration of its even potential presence is obligatory in many countries [US Food Allergen Labelling and Consumer Protection Act of 2004; Regulation (EU) No. 1169/2011]. If not declared, the presence of soybean in the product can cause serious health problems in sensitive people. The assays confirmed the presence of bovine DNA in all spray creams. All the samples were either directly made of bovine cream or milk, or milk protein was added to a mixture of vegetable fats (C8). On the other hand, soybean was detected only in two samples (C1 and C7). The presence of soybean was declared only in sample C7 while sample C1 had no such declaration. The consumption of this spray cream might therefore pose a risk to consumers who would consider it soybean-free.

CONCLUSION

In this study, we have developed HRM assays for fast, accurate and convenient detection of soybean in bovine dairy products and *vice versa* using species specific DNA regions. The assays were able to identify bovine and soy DNA in a number of dairy and soy samples with different matrices, including commercial spray creams. The latter experiment revealed that one of the spray creams had an undeclared content of soybean. As soybean is an important allergenic food, there is a need for development of cost-effective assays for detection of food ingredients so that the competent authorities can ascertain quality and safety of food products available in the market.

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REFERENCES

Berdal K.G., Holst-Jensen A. (2001): Roundup Ready® soybean event-specific real-time quantitative PCR assay and estimation of the practical detection and quantification limits in GMO analyses. European Food Research and Technology, 213: 432–438.

Costa J., Amaral J.S., Grazina L., Oliveira M.B.P.P., Mafra I. (2017): Matrix-normalised real-time PCR approach to quantify soybean as a potential food allergen as affected by thermal processing. Food Chemistry, 221: 1843–1850.

Dalmasso A., Civera T., La Neve F., Bottero M.T. (2011): Simultaneous detection of cow and buffalo milk in mozzarella cheese by real-time PCR assay. Food Chemistry, 124: 362–366.

Espiñeira M., Herrero B., Vieites J.M., Santaclara F.J. (2010): Validation of end-point and real-time PCR methods for the rapid detection of soy allergen in processed products. Food Additives & Contaminants: Part A, 27: 426–432.

Feligini M., Bonizzi I., Cubric Curik V., Parma P., Greppi G.F., Enne G. (2005): Detection of adulteration in Italian mozzarella cheese using mitochondrial DNA templates as biomarkers. Food Technology and Biotechnology, 43: 91–95.

Foucard T., Malmheden Yman I. (1999): A study on severe food reactions in Sweden – is soy protein an underestimated cause of food anaphylaxis? Allergy, 54: 261–265.

Ibáñez C., Acunha T., Valdés A., García-Cañas V., Cifuentes A., Simó C. (2016): Capillary Electrophoresis in Food and Foodomics. In: Schmitt-Kopplin P. (eds): Capillary Electrophoresis: Methods and Protocols. Springer New York, New York, NY, 1483: 471–507.

Jablonski J.E., Moore J.C., Harnly J.M. (2014): Nontargeted detection of adulteration of skim milk powder with foreign proteins using UHPLC–UV. Journal of Agricultural and Food Chemistry, 62: 5198–5206.

Lockley A.K., Bardsley R.G. (2000): DNA-based methods for food authentication. Trends in Food Science & Technology, 11: 67–77.

López-Calleja I., Alonso I.G., Fajardo V., Rodríguez M.A., Hernández P.E., García T., Martín R. (2005): PCR detection of cows' milk in water buffalo milk and mozzarella cheese. International Dairy Journal, 15: 1122–1129.

López-Calleja I., González I., Fajardo V., Martín I., Hernández P.E., García T., Martín R. (2007): Real-time TaqMan PCR for quantitative detection of cows' milk in ewes' milk mixtures. International Dairy Journal, 17: 729–736.

Mafra I., Ferreira I.M.P.L.V.O., Faria M.A., Oliveira B.P.P. (2004): A novel approach to the quantification of bovine milk in ovine cheeses using a duplex polymerase chain reaction method. Journal of Agricultural and Food Chemistry, 52: 4943–4947.

https://doi.org/10.17221/125/2020-CJFS

Mayer H.K., Bürger J., Kaar N. (2012): Quantification of cow's milk percentage in dairy products – a myth? Analytical and Bioanalytical Chemistry, 403: 3031–3040.

Nwaru B.I., Hickstein L., Panesar S.S., Roberts G., Muraro A., Sheikh A. (2014): Prevalence of common food allergies in Europe: A systematic review and meta-analysis. Allergy, 69: 992–1007.

Phipps R.H., Beever D.E., Humphries D.J. (2002): Detection of transgenic DNA in milk from cows receiving herbicide tolerant (CP4EPSPS) soyabean meal. Livestock Production Science, 74: 269–273.

Phipps R.H., Deaville E.R., Maddison B.C. (2003): Detection of transgenic and endogenous plant DNA in rumen fluid, duodenal digesta, milk, blood, and feces of lactating dairy cows. Journal of Dairy Science, 86: 4070–4078.

Rani A., Sharma V., Arora S., Lal D., Kumar A. (2015): A rapid reversed–phase thin layer chromatographic protocol for detection of adulteration in ghee (clarified milk fat) with vegetable oils. Journal of Food Science and Technology, 52: 2434–2439.

Rea S., Chikuni K., Branciari R., Sangamayya R.S., Ranucci D., Avellini P. (2001): Use of duplex polymerase chain reaction (duplex–PCR) technique to identify bovine and water buffalo milk used in making mozzarella cheese. Journal of Dairy Research, 68: 689–698.

Ren Q.R., Zhang H., Guo H.Y., Jiang L., Tian M., Ren F.Z. (2014): Detection of cow milk adulteration in yak milk by ELISA. Journal of Dairy Science, 97: 6000–6006.

Rott M.E., Lawrence T.S., Wall E.M., Green M.J. (2004): Detection and quantification of roundup ready soy in foods by conventional and real-time polymerase chain reaction. Journal of Agricultural and Food Chemistry, 52: 5223–5232.

Sakaridis I., Ganopoulos I., Argiriou A., Tsaftaris A. (2013a): A fast and accurate method for controlling the correct labeling of products containing buffalo meat using High Resolution Melting (HRM) analysis. Meat Science, 94: 84–88.

Sakaridis I., Ganopoulos I., Argiriou A., Tsaftaris A. (2013b): High resolution melting analysis for quantitative detection of bovine milk in pure water buffalo mozzarella and other buffalo dairy products. International Dairy Journal, 28: 32–35.

Song H., Xue H., Han Y. (2011): Detection of cow's milk in Shaanxi goat's milk with an ELISA assay. Food Control, 22: 883–887.

Walker J.A., Hughes D.A., Anders B.A., Shewale J., Sinha S.K., Batzer M.A. (2003): Quantitative intra-short interspersed element PCR for species-specific DNA identification. Analytical Biochemistry, 316: 259–269.

Wu R., Wang Z., Fung Y.S., Peng Seah D.Y., Yeung W.S.B. (2014): Assessment of adulteration of soybean proteins in dairy products by 2D microchip-CE device. Electrophoresis, 35: 1728–1734.

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