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Detection of pea in food by real-time polymerase chain reaction (PCR)

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Abstract A qualitative 5'-nuclease real-time PCR-based method for the detection of pea (*Pisum sativum*) in food is described. The method consists of DNA isolation by chaotropic solid phase extraction and the subsequent PCR with pea-specific primers and a TaqMan fluorescent probe. The primers and the probe are oriented to the chloroplast DNA intron located between *trnL* and *trnF* exons encoding for tRNA. The analytical parameters of the method were inclusivity 100%, exclusivity 100% and the detection limit of 0.11 ± 0.07 ng of pea DNA corresponding to 12 ± 7 diploid pea genome copies. Using a set of model meat pâtés with defined pea contents, a matrix-related detection limit of 0.05% was determined and a linear calibration line was constructed. The presented analytical method was useful for qualitative detection or semiquantitative determination of pea in food products. The method was relatively fast because the analysis could be performed in one working day.

Keywords Pea · Allergen · Polymerase chain reaction (PCR) · Meat

Introduction

Pea (*Pisum sativum*) is an important legume which is widely cultivated in Europe. Because of its high protein contents and high productivity in temperate European climate conditions, pea belongs to the most effective protein sources for food and feed purposes [1]. Further utilization of pea in the food sector has been promoted by the development of pea protein concentrates, which can be used, e.g. as protein supplements in bakery products or a substitute for meat in meat products [2, 3]. However, use of pea in food products may be considerably restricted by its allergenicity, which has been attributed mainly to proteins vicillin

and convicillin [4, 5]. To facilitate the effective control of the use of pea in food products, an appropriate analytical method is required. Here we present a rapid, sensitive and specific method for the detection of pea in food products, that is based upon 5'-nuclease real-time polymerase chain reaction (PCR).

Materials and methods

Plant and food materials

Seeds of pea varieties and other legumes were obtained from gardening shops in Bratislava, Slovakia. Standard samples of flour from pea, wheat, barley, rye, potato starch and standard pâté samples with defined pea contents were obtained from Bundesanstalt für Fleischforschung, Kulmbach, Germany. The standard samples were prepared in frames of a European research project "Development of quantitative and qualitative molecular biological methods to identify plant and animal species in foods (Molspec-ID)" and detailed information on them is available from Molspec-ID Online Database at www.bats.ch/molspec. Food samples were obtained from grocery stores in Bratislava, Slovakia.

DNA extraction

Seeds were soaked in distilled water for 24 h prior to DNA extraction. DNA from seeds, flour and food samples was isolated by chaotropic solid phase extraction using the GeneSpin kit (GeneScan, Teltow, Germany). To obtain the leaf material, seeds were allowed to germinate for 1–2 weeks. DNA from leaves was isolated by DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA concentration was determined by UV-spectrometry.

Determination of sensitivity

For estimation of the detection limit, a series of decimal dilutions (1 ml each) of pea flour-DNA in distilled water

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Table 1 Primers and fluorescent probes used in PCR

Designation	Sequence (5'–3')	Specificity	Reference
PS1for	caaaagccaattaaattccctcat	Pea	www.bats.ch/molspec
PS1rev	gacgcaccatcctaattttaagaaa	Pea	www.bats.ch/molspec
PS1Pb	FAM–ttgatggccctttttgatttagtgacatagactc–TAMRA	Pea	www.bats.ch/molspec
TR03	tctgcctatcaactttcgatgga	Eukaryotic	[6]
TR04	aatttgcgcgctctgccttctt	Eukaryotic	[6]
TRPb	FAM–ccgtttctcaggctcctctccggaatgaacc–TAMRA	Eukaryotic	This article

were used. A practical detection limit of pea content in meat-based samples was estimated using a set of model meat pâtés with defined pea contents. DNA isolated from meat pâtés was adjusted to a concentration of 40 ng/μl.

Polymerase chain reaction

PCR reaction was performed in a volume of 25 μl. Each reaction contained 5 μl of the template DNA, 1.5 U DNA polymerase HotStarTaq (Qiagen), 1× reaction buffer for DNA polymerase, 2.5 mmol/l MgCl₂ and 200 μmol/l dNTP mixture (Invitrogen, Carlsbad, Calif., USA). Detection of pea was performed with 500 nmol/l PS1for primer, 500 nmol/l PS1rev primer and 400 nmol/l PS1Pb probe (Table 1). Sequences of these primers and probe, which align to trnL–trnF intergenic spacer region in the pea chloroplast genome, were obtained from the Molspec-ID Online Database at www.bats.ch/molspec. The length of the amplification product was 92 bp. Amplifiability of individual samples was checked in separate PCR reactions using universal eukaryotic PCR primers TR03 and TR04 ([6], 500 nmol/l each) and a TRPb probe (200 nmol/l, Table 1). The length of the amplification product was 137 bp.

PCR was performed in a PTC-200 thermal cycler with an optical unit Chromo 4 (MJ Research, Waltham, Mass., USA). The PCR programme consisted of the initial denaturation at 94 °C for 15 min followed by 40 cycles of denaturation at 94 °C for 15 s and annealing with polymerization at 60 °C for 60 s. The fluorescence was measured in channel 1. The threshold cycle C_T was calculated for individual samples by the internal software of the cycler using the manual threshold setting at a fluorescence value of $10^{-1.8}$. For the interpretation of the results, all C_T values below 29 were considered as positive whereas all C_T values above 29 were treated as negative.

Results and discussion

The PCR method was used to analyse plant material from pea, other legumes and taxonomically unrelated agricultural plants which may be present in food products together with or instead of pea. All seven pea samples gave positive results, while all plant materials other than pea (23 samples) gave either no amplification signal or an amplification signal at $C_T \geq 29$ (Fig. 1, Table 2). This meant that no false negative and no false positive results were obtained

(inclusivity, 100% and exclusivity, 100%, in terms of ISO 16140 [7]). The amplification of the non-target legume DNA, which was observed in certain samples at $C_T \geq 29$ and was probably caused by the presence of a partially homologous target in these samples, could theoretically be avoided by increasing the annealing temperature. However, we decided rather to shift the positivity threshold to $C_T = 29$ and to retain the practical advantage of using a universal

Table 2 Species analysed, the results of PCR specific for pea and the amplification control

Species	Cultivar or specification of the sample	Result on pea content ^b
<i>Pisum sativum</i>	Ctirad (leaves)	+
<i>Pisum sativum</i>	Moravan (leaves)	+
<i>Pisum sativum</i>	Gloriosa 1 (leaves)	+
<i>Pisum sativum</i>	Gloriosa 2 (leaves)	+
<i>Pisum sativum</i>	Gloriosa 3 (leaves)	+
<i>Pisum sativum</i>	Progress No. 9 (leaves)	+
<i>Pisum sativum</i>	Zázrak z Kelvedonu (leaves)	+
<i>Arachis hypogaea</i>	(roasted seeds)	–
<i>Cicer arietinum</i>	(leaves)	–
<i>Cicer arietinum</i>	(seeds)	–
<i>Glycine max</i>	(leaves)	–
<i>Hordeum vulgare</i>	(barley flour)	–
<i>Juglans regia</i>	(shelled walnut)	–
<i>Lens culinaris</i>	(leaves)	–
<i>Phaseolus vulgaris</i>	white butter beans (leaves)	–
<i>Phaseolus vulgaris</i>	white butter beans (seeds)	–
<i>Phaseolus vulgaris</i>	Katka (leaves)	–
<i>Phaseolus vulgaris</i>	Katka (seeds)	–
<i>Phaseolus vulgaris</i>	Maxidor (leaves)	–
<i>Phaseolus vulgaris</i>	Maxidor (seeds)	–
<i>Secale cereale</i>	(“rye 3” flour) ^a	–
<i>Solanum tuberosum</i>	(potato starch) ^a	–
<i>Triticum aestivum</i>	(“wheat 1” flour) ^a	–
<i>Triticum aestivum</i>	(“wheat 2” flour) ^a	–
<i>Vicia faba</i>	(leaves)	–
<i>Vicia faba</i>	(seeds)	–
<i>Vigna angularis</i>	(seeds)	–
<i>Vigna mungo</i>	(leaves)	–
<i>Vigna mungo</i>	(seeds)	–
<i>Zea mays</i>	(maize flour)	–

^aobtained from Bundesanstalt für Fleischforschung, Kulmbach, Germany

^b(+): positive result; (–): negative result

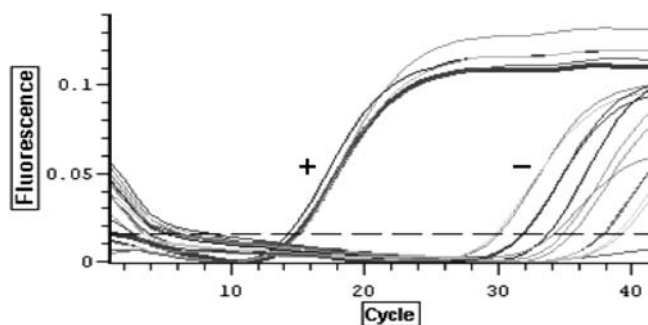


Fig. 1 A record of real-time PCR with undiluted plant DNA from pea and other legumes (Table 2), illustrating the distinction between positive results for pea samples (+) and negative results for other legumes (–)

amplification programme which is suitable for the parallel analysis of various targets in one PCR run.

The positivity threshold at $C_T=29$ was established based on amplification of freshly isolated undiluted DNA from leaves of *Vicia faba*, *Lens culinaris* and *Cicer arietinum*, which occurred at or slightly above this C_T . This value reflects a maximal amplification of the unwanted homologue of pea *trnL-trnF* chloroplast intergenic spacer region in non-pea legumes. In real food samples, the false positive amplification, if any, occurs much later (at higher C_T), because (i) instead of leaves, seeds are usually used in food, which contain much lower amount of chloroplast DNA than leaves, (ii) DNA of legumes in food is diluted by other food components and (iii) DNA in processed foods is usually partially degraded. Based on these reasons, we considered $C_T=29$ a reliable threshold level and amplification occurring at $C_T<29$ was treated as positive.

Using DNA isolated from pea flour, the lowest amount of pea DNA from which the target DNA could be amplified was 0.05 pg DNA. However, when $C_T=29$ was used as the positivity threshold, the average detection limit of four independent experiments (not shown) increased to 0.11 ± 0.07 ng of pea DNA, i.e. 12 ± 7 diploid genome copies [8]. Thus, in our conditions, we were confident to detect up to 0.11 ± 0.07 ng of pea DNA, although improving the PCR specificity by increasing the annealing temperature could theoretically lead to a detection limit down to 0.05 pg pea DNA.

When DNA samples from model pâtés with defined pea contents were analysed, the sample with 0.01% was analysed as positive in some replicates, whereas in some replicates it was reported as negative, because the C_T values were close above or below the positivity threshold. However, 0.05% pea content was reproducibly detected as positive (Fig. 2, Table 2). Thus, the matrix-related detection limit of pea in these meat pâtés was between 0.01 and 0.05%. An important note to the empirical detection limit observed in the model pâtés is, that it is only achieved when at least 200 ng of DNA per reaction is available. This amount of amplifiable DNA cannot be always obtained from highly processed food samples (Table 2).

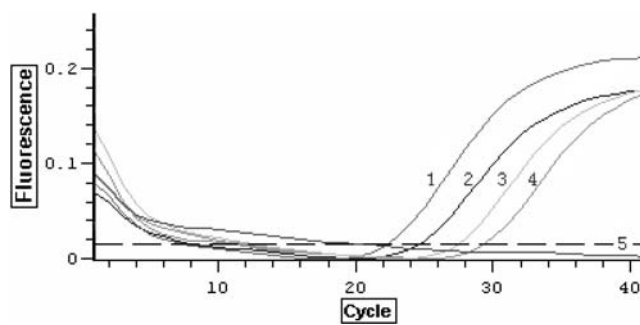


Fig. 2 Real-time PCR analysis of the series of model pâtés with the pea contents of 1% (1), 0.2% (2), 0.05% (3), 0.01% (4) and 0% (5)

A collection of 34 food samples were analysed for the pea content (Table 3). In most cases, results of the detection of pea DNA were in agreement with the declaration of pea content on the label of individual food products. However, two infant formulas, which were supposed to contain pea, did not produce positive results. This discrepancy was probably caused by low amplifiable DNA contents in these highly-processed samples, as suggested by negative results of the amplification control. On the other hand, three samples, which had no declaration of pea contents on their labels, contained pea DNA. These included ham mousse, two dehydrated soups and a poultry salad (Table 3).

The method was also tested with regard to its suitability for (semi)quantitative determination of pea in food products. When DNA samples extracted from model pâtés with defined pea contents were analysed, a linear relationship between the pea contents and the threshold cycle was determined (Fig. 3). These results demonstrate that the method is, in principle, suitable for quantitative applications. However, for true quantification, individual calibration for various types of food products, which differ in the composition and the degree of processing, will be necessary.

As demonstrated, the presented analytical method was useful for sensitive and selective qualitative detection or

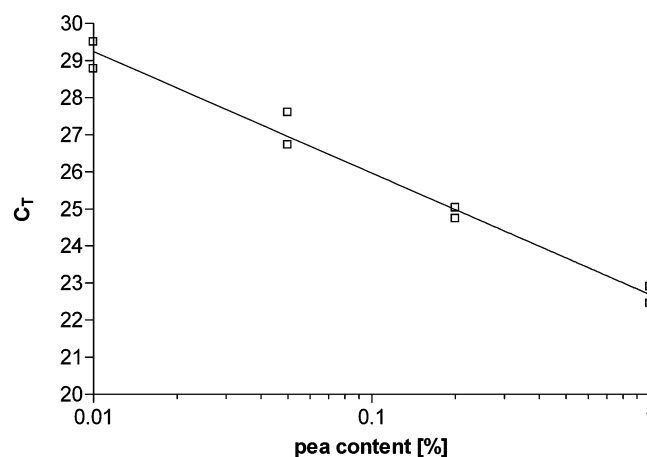


Fig. 3 Relationship ($y = -3.279 \log x + 22.69$; $r^2 = 0.9809$) between the pea contents in model pâtés and the threshold cycle C_T . DNA amount was normalized to 200 ng per reaction

Table 3 Food products analysed, the results of PCR specific for pea and the amplification control

Sample	Country of origin	Declared pea content ^b	Result on pea content ^b
Model pâté S11 ^a	Germany	—	—
Model pâté S13 ^a	Germany	+, 0.01%	—
Model pâté S14 ^a	Germany	+, 0.05 %	+
Model pâté S10 ^a	Germany	+, 0.2 %	+
Model pâté S12 ^a	Germany	+, 1%	+
Pâté 1	Czech Republic	—	—
Pâté 2	Czech Republic	—	—
Pâté 3	Belgium	—	—
Pâté 4	Slovakia	—	—
Vegetarian spread	Slovakia	—	n.d.
Ham mousse 1	Slovakia	—	—
Ham mousse 2	Slovakia	—	+
Infant formula 1	Hungary	—	—
Infant formula 2	Hungary	—	n.d.
Infant formula 3	Hungary	+, 10%	n.d.
Infant formula 4	Poland	—	—
Infant formula 5	Poland	+	n.d.
Infant formula 6	Poland	—	—
Dehydrated soup 1, goulash	Czech Republic	—	—
Dehydrated soup 2, pea with bacon	Czech Republic	+, 57%	+
Dehydrated soup 3, lentil	Czech Republic	—	+
Dehydrated soup 4, goulash	Czech Republic	—	—
Dehydrated soup 5, goulash	Czech Republic	—	+
Dehydrated soup 6, pea with bacon	Czech Republic	+, 49.1%	+
Dehydrated soup 7, chicken	Czech Republic	—	—
Dehydrated soup 8 “French”	Slovakia	—	—
Dehydrated soup 9, tripe	Slovakia	—	—
Dehydrated soup 10, bean	Slovakia	—	+
Dehydrated soup 11, pea	Slovakia	+, 44%	+
Dehydrated soup 12, green pea	Slovakia	+, 41%	+
Dehydrated soup 13, noodle and vegetable	Vietnam	—	—
Salad 1, poultry	Slovakia	—	+
Salad 2, “Vlašský”	Slovakia	+, picture only	+

^aobtained from Bundesanstalt für Fleischforschung, Kulmbach, Germany

^b(+): positive result; (—): negative result; n.d.: not determined because of insufficient DNA

semiquantitative determination of pea in food products. The method was relatively fast because the analysis could be performed in one working day.

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