doi: 10.17221/374/2016-CJFS

Detection of PCR Inhibition in Food and Feed with a Synthetic Plasmid

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Abstract

Sovová T., Křížová B., Drábková L., Ovesná J. (2017): Detection of PCR inhibition in food and feed with a synthetic plasmid. Czech J. Food Sci., 35: 160–164.

We present a successful use of the plasmid inhibition detection and DNA isolation protocol optimisation for four food/feed samples in qPCR analysis of the sequence coding for chloroplast tRNA-Leu: two meat meal samples and two samples made of cranberries (jam and dried fruit). The quantitative real-time polymerase chain reaction (qPCR) can be inhibited by various substances and the DNA content in the sample can be underestimated. It is necessary to identify the PCR inhibition and choose an optimal DNA isolation protocol to correctly evaluate the sample. In a previous study, we have developed an assay using plasmid DNA carrying a non-homologous random sequence identifying possible inhibitors in qPCR in food/feed samples. The plasmid assay allowed to effectively reveal the PCR inhibition in all of the different sample matrices and to choose an optimal DNA isolation protocol.

Keywords: DNA isolation; food analysis; food quality; PCR inhibitor

The quantitative real-time polymerase chain reaction (qPCR) has become a fundamental method used for DNA analysis in many fields, including food science and technology, where it has become a crucial technique used in food quality and safety control (Renault *et al*. 2004; Deer *et al*. 2010; Rodríguez-Lázaro & Hernandéz 2013). Real-time PCR can be used to identify human pathogens, to quantify genetically modified organisms (GMOs), or to check correct labelling of food and feed (Renault *et al*. 2004; Deer *et al*. 2010). Even though qPCR is a very sensitive and precise technique, it can be hindered by the presence of the so-called PCR inhibitors or less common PCR enhancers (Hoorfar *et al*. 2004; Hartman *et al*. 2005; Nordstrom *et al*. 2007). Those substances, when present in the PCR mixture, interfere with the PCR via diverse mechanisms and can result in under- or overestimation of the content of the target DNA sequence in the sample (Hartman *et al*. 2005; Nordstrom *et al*. 2007). Inhibiting substances can originate from the sample itself or be introduced during its processing (Bickley & Hopkins 1999; Hartman *et al*. 2005; Cook *et al*. 2013). However, in certain cases, even the most careful manipulation and/or optimisation of the sample processing protocol cannot fully prevent the occurrence of inhibiting substances in analysed samples. Therefore, for a correct interpretation of the results, it is necessary to identify the presence of the inhibitors (Lübeck *et al*. 2003; Deer *et al*. 2010). One of the possible approaches is to perform a serial dilution of the sample, which is however quite time-consuming and requires a large quantity of PCR reagents. Another possibility is to use an amplification control consisting in adding a known amount of an exogenous nucleic acid into the reaction mixture; the analysed amount of this control is compared with the added amount and the efficiency of the PCR reaction and presence of possible inhibitors can be evaluated (Hoorfar *et al*. 2004; Hartman *et al*. 2005; Nordstrom *et al*. 2007).

Supported by the Ministry of Agriculture of the Czech Republic, Projects No. RO0417 and No. QJ1530272.

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Food and feed samples can present as problematic in terms of DNA analysis as they can represent rich sources of PCR inhibitors (Lübeck *et al*. 2003; Deer *et al*. 2010). It is therefore necessary to check for the presence of PCR inhibitors and, if those are present, to choose an optimal DNA isolation protocol to ensure that the content of such substances are at a level that would not hinder the subsequent analysis. In a previous study, we have developed an assay using a custom plasmid DNA carrying a non-homologous random sequence for use as an amplification control to identify and quantify possible inhibitors in qPCR in food and feed samples (Sovová *et al*. 2016). Here we present a successful use of the assay to identify PCR inhibition in four real food and feed samples in an assay for the presence of the sequence coding for chloroplast tRNA-Leu.

MATERIAL AND METHODS

Samples and DNA isolation. Four food and feed samples were used in this study: lamb meal, beef stomach meal, dried cranberries, and cranberry jam. The feed samples were obtained directly from the producer; the cranberry samples were purchased in a local supermarket.

Two different DNA isolation methods were used for each sample. The feed samples were isolated using the cetyltrimethylammonium bromide (CTAB) method according to the ISO 21571:2005 standard and the NucleoSpin® Food kit (NS; Macherey-Nagel, Germany). The cranberry samples were isolated using the NS kit and the DNeasy Plant Mini Kit (DPM; QIAGEN, Germany). Isolation using the kits was performed according to the manufacturers' instructions without any modifications. The concentration and purity of the obtained DNA samples were determined using UV spectrophotometry (NanoPhotometer P300; Implen, Germany).

Quantitative PCR. All of the samples were analysed for the presence of the sequence coding for chloroplast tRNA-Leu. The sequences of assay oligonucleotides are presented in Table 1. Both primers were purchased from Generi Biotech Ltd. (Czech Republic); the Taqman FAM-labelled fluorescent probe with a non-fluorescent quencher was purchased from KRD Ltd. (Czech Republic). The qPCR reaction was carried out in a volume of 25 µl using the following reaction mixture components (final concentrations): 1 × TaqMan 2 × Universal PCR Master Mix (Thermo Fisher Scientific, USA), 300 nM of the forward and reverse primers, and 200 nM of the probe. The remainder of the reaction mixture volume consisted of 5.5 µl of nuclease free water (NFW; Sigma, Germany) and 5 µl of the DNA sample. The sample was added at the concentration of 20 ng/µl. DNA isolated from soybeans using the above-mentioned CTAB protocol was used as a control. The qPCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s with fluorescence acquisition after each 60°C step. All qPCR assays were performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems, USA) and data were analysed using the SDS Software v2.2.2 (Applied Biosystems, USA).

Detection of PCR inhibition. To detect the inhibition, a newly developed recombinant plasmid DNA carrying a synthetic DNA sequence (pCRI-inh1) was used. A 78-bp long DNA sequence was designed in silico and cloned into a plasmid vector. The design, validation, and optimisation of the plasmid and the assay were described in Sovová *et al*. (2016). The inhibition was identified by amplifying the sequence

Table 1. Assay oligonucleotides

Assay	Oligo	Sequence (5' to 3')	Source
Plasmid (pCRI-inh1)	amplified segment	AGGCGGCTAGATGTGTAACGCTTCAATTTCGAACAATGTAC-CAGGTCGGCGCGATTATTTCTAGCCCGACAGGTCTGT	Sovová *et al*. (2016)
	forward	AGGCGGCTAGATGTGTAACG	
	reverse	ACAGACCTGTCGGGCTAGAA	
	probe	6-FAM-GAACAATGTACCAGGTCGGC-MGBNFQ	
tRNA-Leu	forward	ATTGAGCCTTGGTATGGAAACCT	Taberlet *et al*. (1991)
	reverse	GGATTTGGCTCAGGATTGCC	
	probe	FAM-TTAATTCCAGGGTTTCTCTGAATTTGAAAGTT-TAMRA	

doi: 10.17221/374/2016-CJFS

in the presence of the analysed DNA and comparing the cycle threshold (Ct) value with amplification without the sample DNA (i.e. the control). The synthetic DNA segment and assay oligonucleotides are presented in Table 1. The DNA segment and both primers were purchased from Generi Biotech Ltd. (Czech Republic); the TaqMan FAM labelled fluorescent probe with a non-fluorescent quencher was purchased from Thermo Fisher Scientific (United Kingdom). The qPCR reaction was carried out in a volume of 25 µl using the following reaction mixture components (final concentrations): 1 × TaqMan 2 × Universal PCR Master Mix (Thermo Fisher Scientific, USA), 250 nM of the forward and reverse primers, and 80 nM of the probe. The remainder of the reaction mixture volume consisted of 0.6 µl of NFW, 5 µl of the plasmid stock, and 5 µl of the DNA sample. The sample DNA was added at the concentration of 20 ng/µl. The qPCR conditions were the same as above.

Data analysis. Results are expressed as mean ± standard deviation (SD). The statistically significant differences between experimental groups were determined by one-way ANOVA followed by Tukey's post-hoc test (IBM SPSS Statistics 22). Differences were considered statistically significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

All of the analysed samples were tested for the presence of the sequence coding for chloroplast tRNA-Leu (Table 2). The meat meal samples were destined as ingredients in dog food and were analysed to check for contamination by genetically modified plant material. The animal parts used for the production were not washed and could contain residues of the stomach contents which could contain genetically modified feed. The cranberry samples were analysed to check for the presence of amplifiable plant DNA to confirm the quality of the DNA isolates to later distinguish between the different *Vaccinium* species in product authenticity control. The DNA was isolated using a method that is by default used for similar types of matrix in our laboratory (CTAB protocol for feed samples and NS kit for processed food samples). However, the tRNA-Leu assay did not detect the target sequence for either of the samples and thus, the samples were checked for the presence of PCR inhibitors using the plasmid assay. The results showed an important inhibition for all four samples (Table 2) and therefore the extraction protocol needed to be optimised.

In the case of the meat samples, the DNA was at first isolated using the CTAB protocol. This protocol has been widely used to isolate DNA from mostly plant material due to the availability and low cost, and is often accepted as a 'gold standard' among DNA isolation methods (Olexová *et al*. 2004). However, it has not been proved suitable for the meat meal samples. Animal-based materials contain many different PCR-inhibiting substances [such as collagen (Scholz *et al*. 1998), melanin (Eckhart *et al*. 2000), heme (Al-Soud & Rådström 2001), myoglobin (Bélec *et al*. 1998), or lactoferrin (Al-Soud & Rådström 2001)] that were most probably not sufficiently removed during

Table 2. DNA concentration and purity ranges, and Ct values (mean ± SD, $n = 3$) for tRNA-Leu and plasmid inhibition assays for food and feed samples isolated using CTAB, NucleoSpin® Food kit (NS), or DNeasy Plant Mini Kit (DPM); DNA isolated from soy-beans using CTAB was used as control for the tRNA-Leu assay

Sample	Isolation method	DNA concentration range (ng/µl)	DNA purity range (A260/A280)	tRNA-Leu	Plasmid inhibition analysis
Beef stomach meal	CTAB	730–974	1.88–1.90	n/d	30.60 ± 3.97**
	NS	537–736	1.92–1.97	28.33 ± 0.41	20.00 ± 0.10
Lamb meal	CTAB	646–712	1.86–1.87	n/d	32.83 ± 1.31**
	NS	58–104	1.78–1.81	32.63 ± 0.28	19.88 ± 0.14
Cranberry jam	NS	37–49	0.91–1.01	n/d	27.32 ± 1.73**
	DPM	16–17	0.87–0.91	27.49 ± 0.56	19.82 ± 0.70
Dried cranberries	NS	71–78	0.78–0.90	n/d	35.34 ± 2.86***
	DPM	5–10	1.39–1.43	n/d	23.36 ± 0.30**
Control	CTAB	169–192	1.81–1.82	24.90 ± 0.91	19.79 ± 0.10

Significant differences from plasmid control: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; n/d – not determined

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the CTAB isolation. Even though the purity of the samples was satisfactory, the high Ct values of the plasmid assay showed an important inhibition in both samples. The DNA was afterwards isolated using the NS kit that was also used in other studies for DNA isolation from meat products (Nesvadbová *et al*. 2014; Duarte *et al*. 2015; Houhoula *et al*. 2015). This removed the inhibitory effect of the matrix of both samples when the Ct values of the plasmid assay were not significantly different from the control and the tRNA-Leu sequence was subsequently identified in the sample (Table 2).

The cranberry samples were at first isolated using the NS kit. Even though the amount of DNA isolated from both samples was sufficient, the tRNA-Leu sequence was not detected, which led to an assumption that the DNA was probably too fragmented (Golenberg *et al*. 1996) or the sample contained PCR inhibitors [e.g. polysaccharides or polyphenolic compounds that are abundantly present in vegetable and fruit matrices (Di Pinto *et al*. 2007)]. The plasmid assay confirmed the latter. Similarly, Ganopoulos *et al*. (2011) did not obtain satisfactory results when isolating DNA from cherry jams and biscuits using the NS kit. The DNA was then isolated using the DPM kit which was successfully used in other studies to extract DNA from highly processed food samples, including cherry jam (Di Pinto *et al*. 2007) and fruit juice (Clarke *et al*. 2008). In the case of the jam, the inhibition was eliminated and it was possible to identify the tRNA-Leu sequence in the sample. However, for the dried cranberries, even though the purity of the DNA extract improved as shown by the A260/A280 values, the inhibition was still present and the target sequence was not detected. As stated on the package, the dried cranberries contained various additives, such as citric acid or vegetable oil, which might have an influence on the isolation process and caused the inhibiting substances to persist in the sample. The isolation protocol thus needs to be further optimised, e.g. by incorporating an additional purification step based on solid-phase extraction (SPE) (Green *et al*. 1999; Ganopoulos *et al*. 2011).

In this study, we have successfully used a custom plasmid PCR amplification control in four real food and feed samples. The plasmid effectively revealed important inhibition in the samples which helped to choose an optimal DNA isolation protocol that would minimise the inhibitory substances in the samples to achieve a correct analysis of the samples.

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Received: 2016–09–29
Accepted after corrections: 2017–03–31