Extraction of DNA from different sample types – a practical approach for GMO testing

Ekstrakcija DNA iz različnih vrst vzorcev -praktični pristop za določanje GSO

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Abstract: Current methods based on DNA targets for the detection, identification and quantification of genetically modified organisms (GMOs) involve extraction of the DNA. Different extraction procedures have been developed for the great variety of samples from food, feed, seeds and particular plant parts. This makes the operation of routine analytical laboratories complex and workloads heavy. Here we present a decision-making system, developed over many years of GMO testing on different samples, that result in the application of only a few extraction methods for the majority of samples. Developed decision-making system enables quicker and more cost effective testing of GMOs. In addition, the performance of DNA extraction resulting from the use of the selected extraction methods is presented for use in subsequent testing of GMOs by real time PCR methods. This approach can be used as a model for similar systems based on nucleic acid analysis in food, feed, seeds and plants.

Keywords: Extraction methods, Genetically modified organisms (GMO), Decision-making system, GMO testing, NucleoSpin® Food, Cetyltrimethylammonium bromide (CTAB)

Izvleček: Metode za določanje, identifikacijo in kvantifikacijo gensko spremenjenih organizmov (GSO) temeljijo na zaznavanju značilnih zaporedij DNA, zato je ključni del metode ekstrakcija DNA. Za ekstrakcijo DNA iz različnih vrst vzorcev, kot so živila, krma, semena in deli rastlin, so razviti različni postopki. Delo rutinskih laboratorijev je zato zelo kompleksno in obsežno. Tu predstavljamo sistem odločanja, ki smo ga razvili v mnogih letih testiranja GSO v različnih vzorcih. Z uporabo nekaj izbranih metod ekstrakcije lahko analiziramo večino vzorcev na hitrejši in finančno učinkovitejši način. Dodatno podajamo informacijo o uporabi izbranih ekstrakcijskih metod v povezavi z nadaljnjo analizo GSO s PCR v realnem času. Ta pristop se lahko uporabi kot model za podobne sisteme, ki temeljijo na analizi nukleinskih kislin v živilih, krmi, semenih in rastlinah.

Ključne besede: Metode ekstrakcije, Gensko spremenjeni organizmi (GSO), Sistem odločanja, GSO testiranje, NucleoSpin® Food, Cetiltrimetilamonijev bromid (CTAB)

Introduction

Efficient and reliable extraction of nucleic acids is a prerequisite for obtaining accurate results from molecular analyses such as amplification of specific DNA or RNA sequences by polymerase chain reaction (PCR). The extraction method should yield sufficient DNA of adequate structural integrity and purity, independently of the matrix to which it is applied (Codex Committee On Methods Of Analysis And Sampling 2010). Acceptance criteria applicable to DNA extraction methods were recently defined in the new European Network of GMO Laboratories (ENGL) document on Definition of minimum performance requirements for analytical methods of GMO testing (ENGL 2015).

GMOs are already widespread and countries have different regulations and policies for their management (http://bch.cbd.int/). Many countries have labeling requirements for products containing GMOs, demanding methods for their detection and for their analysis in order to control approved and unapproved GMOs. Some countries have also set thresholds for the unintended presence of GMOs in a product, requiring quantitative analysis of GMOs. Testing for genetically modified organisms (GMOs) is based on multiplication of the genetic elements characteristic of the GMOs, therefore a DNA extraction procedure is crucial (Žel et al. 2012). Only DNA of certain quality is amplifiable in further multiplication using molecular methods. Real-time PCR is the technology of choice for detecting GMOs, especially when quantitative analyses are needed (qPCR) (Žel et al. 2012), even PCR is also used by some laboratories. Other methods, such as isothermal multiplication and digital PCR, that are under rapid development, are also dependent on a good quality extraction method (Milavec et al. 2014).

GMOs have been detected in various samples – food, feed, seeds and plants from the field. Each sample may contain only one ingredient, *e.g.* maize seeds, or a number of ingredients, such as feed composed of a variety of grains, *e.g.* maize, rice and soybean. Additionally, particular processed samples, such as corn flakes, can differ, depending on the manufacturing procedure and the DNA can be present in low amounts and also degraded. As early as 2006, Cankar et al. pointed out that extrac-

tion technique and sample properties have a crucial influence on the results of GMO detection and quantification (Cankar et al. 2006). Molecules of plant origin or from other sources, even components of DNA extraction solutions can influence PCR reactions. During GMO quantification standard curve using certified reference material is used, therefore similarity of PCR efficiency for the sample and certified reference material is a prerequisite for exact quantification. The appropriate extraction method has to be determined for each sample type. Detailed guidelines have been reported for validating the extraction methods applied prior to PCR examination of food and feed products for the presence of genetically modified material (Waiblinger and Grohmann 2014; ENGL 2011).

Selection of methods for DNA extraction and purification is mostly done on experience-based choice of the user, as noted in ISO 21571:2005 that deals with nucleic acid extraction for detecting GMOs (ISO 21571:2005; ISO 21571:2005/ A1 2013). Some extraction methods have already been proposed in this standard, together with their scope and examples of samples for each method. Specific extraction methods have been proposed for challenging samples such as pollen from honey (Waiblinger et al. 2012), soybean lecithin (ISO 21571:2005/A1 2013*)* or choline chloride (Sacco et al. 2014). The influence of extraction method and of sample characteristics on the quality and yield of DNA extracted from different samples has been reviewed (Gryson 2010; Demeke and Jenkins 2010). The suitability of different extraction methods for different samples, the effects of sample processing, the presence of PCR inhibitors in the extraction reagents and in the samples themselves are reviewed, together with the theory behind the effects.

One extraction method can be more suitable for a particular sample than another, but it would be very challenging to test all available methods on all samples. It is worthwhile to mention also that there are different quantification procedures for quantification of extracted nucleic acids however, the methods produce different results, making it unwise to try to compare data obtained with the different methods (Bustin et al. 2009).

Using different extraction methods for different samples is also time and cost wasting for labora-

tories testing many samples daily. It is therefore essential to set up, in the laboratory, a manageable decision-making system that can support selection of the extraction method appropriate for an individual sample.

Here we present a decision-making system for selecting extraction methods for GMO testing on different samples – food, feed, seeds and whole plants. It has been developed over many years of GMO testing at the National Institute of Biology, Ljubljana, where various extraction methods have been tested for individual samples. Additionally, and in line with the decision-making system, data relating the extraction method appropriate for further testing of GMOs with real time PCR to a given sample type, based on practical experience, are tabulated.

Materials and methods

Samples

Samples were taken from routine analyses, including seeds/grains, plant leaves and various food and feed samples (for details see also Tab. 1-6). Each sample (*e.g.* soybean flour, maize corn flakes etc.) was tested at least 5 times from independent consignments.

Homogenization

Most of the samples (seeds, feed, soybean meal, choline chloride, rice, and pasta) were ground with a Retsch ZM200 rotor mill. Some (usually with high fat content, *e.g.* oilseed rape seeds) were cooled with liquid nitrogen before grinding with a Retsch GM200 knife mill. Samples such as sausage or tofu were homogenized using a Bioreba HOMEX 6 homogenizer. Leaf samples were prepared by cutting off small pieces from different leaves, then combining and homogenizing them together as one sample with a FASTprep instrument (MP Biomedicals) using 15ml tubes, a ceramic ball and quartz sand.

DNA extraction

200 mg of homogenized sample was used for all isolations, unless stated otherwise.

Different extraction methods were used (see Tab. 1 to 6).

NucleoSpin® Food (Macherey-Nagel) was used as described by the manufacturer. In lysis step more than 200 mg of sample can be used when needed (e.g. when small amounts of DNA in the sample can be expected) with scaled up lysis buffer volumes and in this case, after lysis, double the amount of supernatant was used for further processing.

Cetyltrimethylammonium bromide (CTAB) method with RNase-A and proteinase-K solutions for removing RNA and proteins from the sample was performed as described in ISO 21571, Annex A.3 – Preparation of PCR-quality DNA using the CTAB-based DNA extraction methods (ISO 21571:2005). Removal of lipid components from chocolate and lecithin was performed using hexane prior the first step of the CTAB method (Solfizzo et al. 1998). For samples with low amount of DNA up to 20 g of sample was used and further steps of DNA extraction were done with larger volumes of chemicals.

Samples of oil were first centrifuged and pellet was used for DNA extraction using NucleoSpin® Food (Macherey-Nagel) (Costa et al. 2010).

The DNeasy Plant Mini Kit (Qiagen) was used to extract DNA from plant leaves, as described by the manufacturer.

All extractions from each sample were carried out in duplicate.

qPCR

The quality of extracted DNA was checked by measuring the efficiency of amplification of reference taxon specific genes by qPCR, using appropriate dilutions of both DNA extractions *e.g.* 10x and 100x (Žel et al. 2012), with the exception of stable sample types (for explanation see results section), for which a second dilution was made only for one extraction. Each dilution of DNA was analyzed in duplicate.

Negative control of extraction and environmental control, no template control and positive control were included. The following qPCR methods were applied: for soybean, the method targeting the lectin Le1 gene (*Le1*) (Pauli et al. 2001), for maize, the method targeting the high mobility group protein gene (*hmga*) (Hernandez

et al. 2004); for oilseed rape the method targeting the phosphoenolpyruvate carboxylase gene (*PEP*) (Zeitler et al. 2002); for rice the method targeting the phospholipase D gene (*PLD*) (Mazzara et al. 2006); for potato and tomato the method targeting the potato and tomato specific metallo-carboxypeptidase inhibitor gene (*POT, TOM*) (Hernandez et al. 2003) and, for flax, the method targeting the stearoyl-acyl carrier protein desaturase gene (*SAD*) (Genetic ID NA Inc. 2009).

The method targeting the 18S rRNA gene was used for confirmation of DNA extraction when insufficient reference gene was present (Eukaryotic 18S rRNA Endogenous Control, Life Technologies, Part No.:4319413E).

qPCRs were randomly run on the ABI PRISM® 7900 HT sequence detection system, ViiA™ 7 (both Life Technologies) or LightCycler® 480 System (Roche). Reactions were performed in 10 µl reaction mixture, using 2 µl of diluted DNA, in a 384-well plate. All qPCR assays were performed using TaqMan® Universal PCR Master Mix (Life Technologies, Part No.: 4304437). PCR cycling conditions were set to 2 min at 50°C and 10 min at 95°C followed by 45 cycles of 15 s at 95°C and 1min at 60°C.

Table 1: Extractions used for soybean samples.
Tabela 1: Ekstrakcije uporabljene za vzorce soje.

Sample	Stability of sample	Test portion	DNA extraction method[a]	Elution volume (µl)	Volume of water added after elution (µl)	Cq value obtained on reference gene
Seeds/grains						
grains	stable	200 mg	NucleoSpin® Food	100 µl+100 µl	800 µl	24-27
Food						
flour	stable	200 mg	NucleoSpin® Food	100 µl+100 µl	800 µl	24-27
tofu-soybean curd	stable	500 mg	NucleoSpin® Food	100 µl+100 µl	300 µl	21-23
soybean drink	variable	500 ul - 1 ml	NucleoSpin® Food	100 µl+100 µl	50 µl	> 22 [b]
Frankfurt sausage, sausage, cold meats	variable	500 mg to 1 g	NucleoSpin® Food	50 µl + 50 µl	/	> 22 [b]
flackes, cracker, crispies	variable	200 mg	NucleoSpin® Food	50 µl + 50 µl	/	> 22 [b]
soy steaks, burger, medallion	variable	200 mg to 1 g	NucleoSpin® Food	50 µl + 50 µl	/	> 24 [b]
soy spread	variable	1 g to 5 g	NucleoSpin® Food	50 µl + 50 µl	/	> 23 [b]
soybean meat, soybean peaces,	variable	200 mg	NucleoSpin® Food	50 µl + 50 µl	/	> 23 [b]
soy nuggets	variable	200 mg	NucleoSpin® Food	50 µl + 50 µl	/	> 22 [b]
biscuits	variable	at least 5 g	CTAB	100 µl	/	> 22 [b]
lecithin	Variable	at least 15 g	hexane + CTAB	100 µl	/	> 28 [b]
chocolate	Variable	20 g	hexane + CTAB	100 µl	/	> 34 [b]
Soya desert	variable	10 g	CTAB	100 µl	/	> 23
Feed						
animal feed	variable	200 mg	NucleoSpin® Food	100 µl+100 µl	300 µl	> 22 [b]
soybean meal	stable	200 mg	NucleoSpin® Food	100 µl+100 µl	800 µl	24-27
soy proteins	variable	200 mg	NucleoSpin® Food	100 µl+100 µl	300 µl	> 22 [b]

[a] when more than 200mg was used for the NucleoSpin® Food extraction, the starting procedure was modified as described in Materials and Methods
[b] actual value depends on the DNA content and processing

Table 2: Extractions used for maize samples.
Tabela 2: Ekstrakcije uporabljene za vzorce koruze.

Sample	Stability of sample	Test portion	DNA extraction method[a]	Elution volume (μl)	Volume of water added after elution (μl)	Cq value obtained on reference gene
Seeds/grains						
kernels	stable	200 mg	NucleoSpin® Food	100 μl+100 μl	/	20-24
Food						
flour	stable	200 mg	NucleoSpin® Food	100 μl+100 μl	/	20-24
semolina	stable	200 mg	NucleoSpin® Food	100 μl+100 μl	/	20-24
corn cobs	stable	200 mg to 1g	NucleoSpin® Food	100 μl+100 μl	/	20-24
corn flakes	stable	200 mg	NucleoSpin® Food	50 μl + 50 μl	/	23-25
chips, tortilla chips	variable	200 mg	NucleoSpin® Food	50 μl + 50 μl	/	> 22 [b]
tortilla	variable	200 mg till 2g	NucleoSpin® Food	50 μl + 50 μl	/	> 22 [b]
canned corn	stable	1 g	NucleoSpin® Food	50 μl + 50 μl	/	20-27
popcorn chips	variable	200 mg	NucleoSpin® Food	50 μl + 50 μl	/	> 25 [b]
bread	variable	5 g	NucleoSpin® Food	50 μl + 50 μl	/	> 25 [b]
crunchy muesli	variable	at least 5 g	CTAB	100 μl	/	> 30 [b]
corn gluten	variable	2 g	CTAB	100 μl	/	> 23 [b]
Feed						
feed	variable	200 mg	NucleoSpin® Food	100 μl+100 μl	300 μl	> 22 [b]
Leaves						
maize leaves	stable	200 mg to 1 g	CTAB / DNeasy Plant Mini kit	100 μl / 50 μl + 50 μl	/	20-27 / 24-30

[a]   when more than 200mg was used for the NucleoSpin® Food extraction, the starting procedure was modified
     as described in Materials and Methods
[b]   actual value depends on the DNA content and processing

Table 3: Extractions used for samples with oilseed rape and flax.
Tabela 3: Ekstrakcije uporabljene za vzorce oljne ogrščice in lanu.

Plant species	Sample	Stability of sample	Test portion	DNA extraction method[a]	Elution volume (µl)	Volume of water added after elution (µl)	Cq value obtained on reference gene
	Seeds						
	seeds	stable	200 mg	NucleoSpin® Food	100 µl+100 µl	300 µl	22-26
	Feed						
seed rape	Oilseed cake	variable	200 mg	NucleoSpin® Food	100 µl+100 µl	300 µl	> 23 [b]
	Leaves						
	leaves	stable	200 mg to 1 g	CTAB / DNeasy Plant Mini kit	100 µl / 50 µl + 50 µl	/	20-27 / 24-30
flax	**Seeds**						
	seeds	stable	200 mg	NucleoSpin® Food	50 µl + 50 µl	/	22 - 26

[a] when more than 200mg was used for the NucleoSpin® Food extraction, the starting procedure was modified as described in Materials and Methods

[b] actual value depends on the DNA content and processing

Table 4.: Extractions used for samples containing rice.
Tabela 4: Ekstrakcije uporabljene za vzorce riža.

Sample	Stability of sample	Test portion	DNA extraction method[a]	Elution volume (µl)	Volume of water added after elution (µl)	Cq value obtained on reference gene
Seeds/grains						
grain	stable	200 mg	NucleoSpin® Food	100 µl+100 µl	50µl	22-24
Food						
waffels	variable	200 mg	NucleoSpin® Food	50 µl + 50 µl	/	> 26 [b]
rice drink	variable	1 ml	NucleoSpin® Food	100 µl+100 µl	50µl	> 22 [b]
rice pudding	variabe	10 g	NucleoSpin® Food	50 µl + 50 µl	/	> 26 [b]
spaghetti	variable	200 mg	NucleoSpin® Food	50 µl + 50 µl	/	> 22 [b]
rice cracker	variable	200 mg	NucleoSpin® Food	50 µl + 50 µl	/	> 28 [b]
boiled rice grains	variable	500 mg to 5g	NucleoSpin® Food	50 µl + 50 µl	/	> 26 [b]
rice bread	variable	200 mg	NucleoSpin® Food	50 µl + 50 µl	/	> 24 [b]
choline chloride (rice)	variable	200 mg	NucleoSpin® Food	50 µl + 50 µl	/	> 27 [b]
Feed						
dog food with rice	variable	200 mg	NucleoSpin® Food	50 µl + 50 µl	/	> 24 [b]

[a] when more than 200mg was used for the NucleoSpin® Food extraction, the starting procedure was modified as described in Materials and Methods

[b] actual value depends on the DNA content and processing

Table 5:   Extractions used for samples with potato.
Tabela 5: Ekstrakcije uporabljene za vzorce krompirja.

Sample	Stability of sample	Test portion	DNA extraction method[a]	Elution volume (µl)	Volume of water added after elution (µl)	Cq value obtained on reference gene
Food						
tuber	stable	0,5 g	NucleoSpin® Food	50 µl + 50 µl	/	> 22[b]
potato puree	variable	1 g	NucleoSpin® Food	50 µl + 50 µl	/	> 30[b]
pommes frites	variable	at least 1 g	NucleoSpin® Food	50 µl + 50 µl	/	> 25[b]
croquettes	variable	at least 1 g	NucleoSpin® Food	50 µl + 50 µl	/	> 28[b]
gnocchi	variable	at least 1 g	NucleoSpin® Food	50 µl + 50 µl	/	> 30[b]
potato starch	variable	1g	CTAB	100 µl	/	> 35 (Ct 18S 25)

[a]   when more than 200mg was used for the NucleoSpin® Food extraction, the starting procedure was modified
      as described in Materials and Methods
[b]   actual value depends on the DNA content and processing

Table 6:   Extractions used for samples with tomato.
Tabela 6: Ekstrakcije uporabljene za vzorce paradižnika.

Sample	Stability of sample	Test portion	DNA extraction method[a]	Volume of water added after elution (µl)	Expected Cq value obtained on reference gene
Food					
fresh tomato	stable	500 mg	NucleoSpin® Food	/	23-27
concentrate	variable	1 ml	NucleoSpin® Food	/	> 33[b]
ketchup	variable	1 ml	NucleoSpin® Food	/	> 35[b]
peeled tomato (canned)	variable	1 ml	NucleoSpin® Food	/	> 33[b]
Leaves	stable	200 mg to 1 g	CTAB / DNeasy Plant Mini Kit	/	20-27 /24-30

[a]   when more than 200mg was used for the NucleoSpin® Food extraction, the starting procedure was modified
      as described in Materials and Methods
[b]   actual value depends on the DNA content and processing

Table 7: Comparison of prices of different extraction methods.
Tabela 7: Primerjava cen različnih izolacijskih metod.

	1 sample			3 samples			10 samples		
Extraction method	Working hours	Chemicals[a]	Final price[b]	Working hours	Chemicals	Final price	Working hours	Chemicals	Final price
NucleoSpin® Food	2,25	6	60	2,5	21	58	6,5	66	94
CTAB	4	5	100	6,5	10	100	9	25	100
CTAB with larger volumes	6	8	127	10	20	203	NA	NA	NA
DNeasy	2,25	10	64	2,5	35	73	6,5	110	109

[a] prices in Slovenia
[b] calculated prices expressed as ratio (CTAB is taken as 100%)

Results and discussion

We divided samples in two categories, known and new sample types, depending on our experiences on their performance during extraction procedure with respect to the efficiency of amplification of reference taxon specific genes by qPCR as explained in Material and Methods section. Known sample types are the ones which we tested already many times and we have experiences on the efficiency of extraction and quality of extracted DNA in contrast with new sample types where no experience have been obtained. Some of the known samples (stable sample types) gave repeatable extraction results, even when obtained from different sources or produced by different manufacturers. Others, (variable sample types), are more variable and unexpected results of DNA extraction can be obtained, so the testing procedure for these samples was adapted.

Some of the samples, known or new, can be also challenging samples, usually containing highly degraded DNA or the presence of inhibitors and they are treated differently.

Testing different extraction methods

Over many years of testing, individual samples were tested in our laboratory by different methods – DNeasy Plant Mini Kit (Qiagen, Valencia, CA), Wizard extraction (Promega, Madison, WI), CTAB based extraction, CTAB extraction and GENESpin kit (GeneScan, Freiburg, Germany) (Cankar et al. 2006), and NucleoSpin® Food. The most important parameter for testing quality of extracted DNA was the qPCR efficiency of amplification of reference taxon specific genes, using appropriate dilutions.

Extraction of DNA from known sample types

For known stable food and feed sample types NucleoSpin® Food was the most successful method, producing the expected yield and quality of DNA. DNA was extracted from leaves with the CTAB method or DNeasy Plant Mini Kit as explained later.

For variable sample types, giving different quantities and qualities of the extracted DNA, some were treated with NucleoSpin® Food and others, mostly more processed ones, with the CTAB method. Sometimes NucleoSpin® Food as the first choice does not give expected result, then CTAB method is applied on the sample.

The CTAB method has been used widely for extracting DNA from leaves, seeds/grains and processed food/feed samples [ISO 21571:2005]. It was developed in 1980 (Murray and Thompson 1980) and the various versions or modifications of the CTAB protocol are reviewed in Demeke and Jenkins (2010). The procedure is however time-consuming and uses hazardous chemicals including phenol and chloroform (Demeke and Jenkins 2010). The CTAB method was therefore

not the first choice. The larger amounts of extracted DNA are not a decisive factor, since the yields obtained by NucleoSpin® Food are sufficient for further analyses in most of the samples according to our experiences.

In cases where samples contain only small amounts of DNA, larger test portions can be used for extracting DNA. A limited sample size (*e.g.* 20–200 mg) is generally used for DNA extraction with kits (Demeke and Jenkins 2010), so the CTAB method is more appropriate in the case of large test portions (e.g., >200 mg). NucleoSpin® Food was also used successfully for sample sizes greater than 200 mg, with an adapted starting procedure as described in Materials and Methods.

Experiences for selection of extraction methods for different samples, arranged according to the prior knowledge on the presence of the main plant species ingredient, are described further.

Known sample types are presented in the corresponding Tables 1-6, where it is also indicated whether a sample is stable or variable. Further information on the testing procedures is given on the size of the test portion of the analytical sample used for extraction and on elution volumes. Since the quality and quantity of extracted DNA is checked by measuring the effectiveness of amplification and the quantity of the reference gene, the expected quantification cycles (Cq) values are also provided.

- Soybean (*Glycine max* (L.) Merr. Fabaceae) samples

Stable soybean sample types, like grains, flour, and most of the food variable ones (*e.g.* soybean drink, sausages) can be extracted with NucleoSpin® Food (Tab. 1)**.** Some (*e.g.* biscuits and soya desert) are extracted with CTAB. If the extraction of DNA from variable sample types extracted with NucleoSpin® Food does not result in the appropriate quality or quantity of DNA, the CTAB method is used. Hexane is used as the first step in the CTAB method for lecithin and chocolate samples. It is known that DNA is difficult to extract from lecithin. In our laboratory very different Cq values were obtained for lecithin samples.

NucleoSpin® Food has been used without any problem for all feed samples containing soybean as the prevailing ingredient.

- Maize (*Zea mays* subsp. *mays*, Poaceae) samples

As for soybean, NucleoSpin® Food was used successfully for all unprocessed and for most processed maize-based food samples (Tab. 2). Exceptions are crunchy muesli and corn gluten, for which the CTAB method is used.

NucleoSpin® Food was used without any problem for all feed samples containing maize as the prevailing ingredient.

DNA was extracted from leaves using either CTAB or DNeasy Plant Mini Kit. CTAB gives higher yields but enough DNA for reliable qPCR analyses can be obtained with DNeasy Plant Mini Kit and the extraction is much quicker. DNeasy Plant Mini Kit was used when multiple samples of leaves had to be tested at the same time.

- Oilseed rape (*Brassica napus* L., Brassicaceae) and flax (Linum usitatissimum L., Linaceae) samples

NucleoSpin® Food can also be used for oilseed rape and flax seeds (Tab. 3). Extraction of DNA with NucleoSpin® Food was also successful for samples of rapeseed cake, used as animal feed.

DNA from oilseed leaves is extracted by CTAB or DNeasy Plant Mini Kit as described for maize leaves.

- Rice (*Oryza sativa* L., Poaceae) samples

All rice samples tested so far have been extracted by NucleoSpin® Food (Tab. 4). The presence of rice was also examined in samples of choline chloride, which is used as an additive in feed, rice being used in the manufacturing process as a plant derived carrier. A special schedule was written for testing in choline chloride, where a slightly modified CTAB method was proposed for extracting DNA (Sacco et al. 2014). In accordance with our system we have used NucleoSpin® Food as our primary method and found that it gives satisfactory results.

- Potato (*Solanum tuberosum* L., Solanaceae) sample types

DNA from potato tubers and from most pro-

cessed food can be extracted with NucleoSpin® Food with the exception of potato starch, for which the CTAB method is used (Tab. 5). For potato tubers we use cores from potato heel ends, where there is more DNA than in other parts of the tuber.

• Tomato (*Solanum lycopersicum* L., Solanaceae) samples

DNA from tomato fruits can be extracted by NucleoSpin® Food (Tab. 6). NucleoSpin® Food is also the first choice for more processed tomato samples, such as canned tomato, ketchup or tomato concentrate. Extraction of these challenging sample types is occasionally not effective and CTAB with larger test portions has to be used. However, this approach can fail to extract enough DNA for further testing. DNA from tomato leaves is extracted by CTAB or DNeasy Plant Mini Kit as described above.

Extraction of DNA from new sample types

Experiences from known sample types are used also in the samples not previously tested. The successful use of NucleoSpin® Food suggests it as the first choice for new sample types. The extracted DNA is then analyzed for the reference gene with qPCR. NucleoSpin® Food is then accepted for use if qPCR amplification efficiency and quantity of the extracted DNA were adequate; otherwise the CTAB mehod is applied, sometimes with the addition of hexane, when more lipids are present in the sample. If the CTAB method is not successful the sample is treated as a challenging sample.

Extraction of DNA from challenging samples

Some samples are generally known as very challenging for the extraction of DNA, including those containing lecithin, starch, chocolate, canned tomato or oil, those that are highly processed and/or those in which DNA is present in very low amounts or is degraded. The effect of food processing on plant DNA degradation and PCR-based GMO analysis is reviewed in more detail by Gryson (2010). The samples can vary greatly and inhibitors of PCR reaction may be present. Certain additional steps can be used to minimize inhibition, like the addition of proteinase K to degrade proteins or of hexane to remove lipid

components (Gryson 2010, Solfizzo et al 1998, Terry et al. 2002). When testing the final product is too difficult, it is advisable to ask for raw material, if available, because the extraction of DNA from the raw material is usually more successful.

Extraction of DNA from oil samples is also difficult, because the DNA remains only in the leftovers of plant material present in the oil. Therefore prior centrifugation of the sample, using the sediment for further extraction with NucleoSpin® Food, is recommended and has been used in our laboratory (Costa et al. 2010).

When testing the effectiveness of DNA extraction by a method targeting the reference gene, the possibility exists that the extraction from the sample with more ingredients was effective but that insufficient reference gene was present. Since preferred reference genes are usually chosen on the basis of their being only one copy, the 18S rRNA gene which is present in more copies and characteristic for all eukaryotic cells, can be additionally used to assess the level of DNA extraction.

Decision-making system for selecting extraction methods

A decision-making system was developed, based on DNA extraction results for known and new sample types, taking into account also challenging samples (Fig. 1). The system dictates the use of two extraction methods for most samples, with practical implications for fluent workflow in the testing of many different samples. Most of the samples could be tested using NucleoSpin® Food or the CTAB method, sometimes with modification for challenging samples.

Financial aspects

In general, published protocols proved to be cheaper than commercial kits because they do not depend on costly reagents covered by international patents (Pirodini et al. 2010). On the other hand DNA extraction kits generally operate faster than the CTAB method (Demeke and Jenkins 2010). Some financial comparisons have been made by Smith et al. (2005) concerning methods for extracting DNA from potatoes and potato-derived products; only costs of materials were compared (Smith et al. 2005). No other exact economic
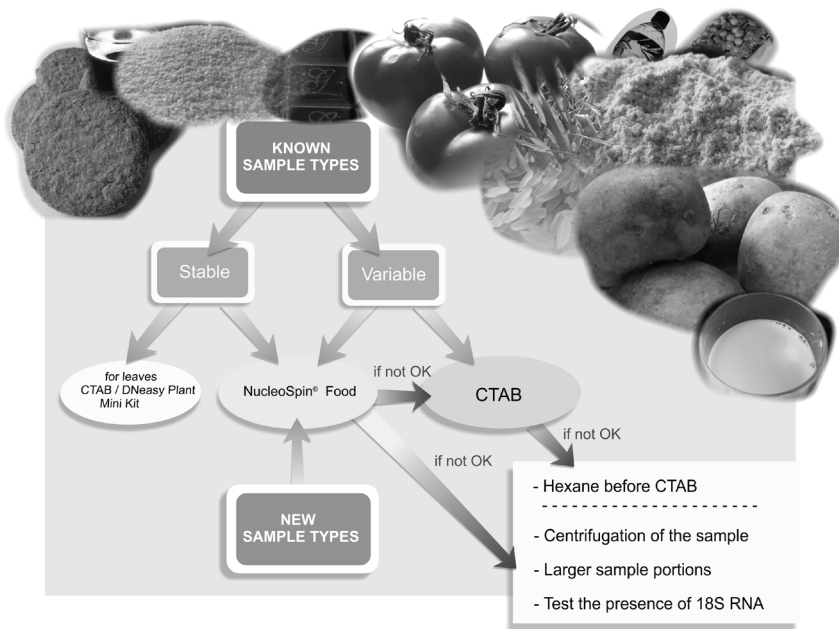
Figure 1:   Decision-making system for selection of DNA extractions.
             Known sample types – tested many times, knowledge on DNA extraction regarding efficiency is known.
             Stable ones give repeatable, while variable ones give unexpected results of DNA extraction.
             New sample types – there is no experience on DNA extraction.
Slika 1:   Sistem odločanja za izbor metode za ekstrakcijo DNA.
             Poznani vzorci - testirani večkrat, poznana je uspešnost ekstrakcije DNA.
             Stabilni vzorci – analiza da pričakovan rezultat, medtem ko je pri variabilnh vzorcih lahko nepričakovan
             rezultat.
             Novi vzorci - zanje ni izkušnje glede uspešnosti ekstrakcije DNA.

evaluations have been published. The financial aspect of the methods selected by our decision-making system follows.

As noted above, NucleoSpin® Food and the CTAB method were used for extracting DNA from most samples. Additionally, the DNeasy Plant Mini Kit was used for extracting DNA from leaves. The number of samples tested at the same time influences the final price; one, three and ten samples were tested in parallel – only the CTAB method using larger volumes is limited to test maximum of three samples at the same time. Working hours, chemicals and final prices were each compared, being expressed as ratios, with CTAB being taken as 100% (Tab. 7). Comparison of the final costs of all the methods showed that NucleoSpin® Food is the most cost effective, especially on account

of the least number of working hours required to perform extractions. Independently of the extraction method used, the price decreases with increasing number of samples on account of the reduced working hours per sample. With more samples tested in parallel, final prices became more comparable.

Future perspectives

The laboratories dealing with many different samples daily need efficient system for extraction of DNA from these samples to enable high through-put and reliable DNA testing results. Developed decision-making system enables organized and supportive structure for quicker decisions in daily workflow in the laboratory.

Besides experiences described from testing of GMOs in our laboratory, further possibilities exist for more efficient operation of the DNA extraction step in testing procedures. The introduction of automation of DNA extraction using magnetic beads should further speed the process and result in higher throughput (Guertler et al. 2014).

The modular approach, declaring individual steps of the detection procedure, like DNA extraction and qPCR as independent modules, is commonly accepted in the detection of GMOs, but not in all other areas using nucleic acid based testing (Bellocchi et al. 2010; Holst-Jensen and Berdal 2004). In GMO detection the ratio between transgene and reference gene is used. In other areas, however, absolute quantification is needed, so it is even more important that extraction methods used in different laboratories are comparable, therefore decision-making systems can be used also for the purposes of standardization.

New technologies can also enable amplification of DNA without prior intensive extraction, as was shown for leaf and seed tissues of MON 810, using loop-mediated isothermal amplification (LAMP) (Lee et al. 2009) or even qPCR (Mano et al. 2014) and digital PCR (Pavšič et al. 2015) giving further possibilities for even more standardized procedures of testing without influences of the extraction step.

Conclusions

The decision-making system for selecting extraction methods described here enables the use of tested methods for most samples. The advantage of such a system is that different samples coming to the laboratory can be processed in parallel using the same method, thus resulting in rationalization of the workflow, labor and cost. In consequence, fewer methods need to be verified or validated and the education of personnel is more rational. The tables presented in this paper, which relate extraction to sample type, can also be useful for other laboratories testing similar sample types. The decision-making system presented here can also contribute to harmonizing the extraction steps for GMO detection used in different laboratories. The decision-making system can also be adapted for other areas using nucleic acids testing in food, feed, seeds and plants.

Povzetek

Pri analizah živil, krme, semen in rastlin, ki temeljijo na določanju nukleinskih kislin, se uporablja različne metode ekstrakcije DNA. Takšen primer je tudi določanje gensko spremenjenih organizmov (GSO), kjer izvajamo analize na zelo različnih vrstah vzorcev, od rastlin ali semen, do zelo procesiranih vzorcev kot so kosmiči ali čokolada, ki se med seboj lahko zelo razlikujejo, npr. zaradi različnih načinov obdelave pri proizvodnji ali različnih dodatkov ipd. Celo vzorci enake sestave se lahko med različnimi proizvajalci razlikujejo glede kvalitete DNA, ki jo vsebujejo. Razvite so različne metode za ekstrakcijo DNA, ki delujejo uspešno pri nekaterih vrstah vzorcev, pri drugih pa ne. Zato je delo v laboratorijih, ki analizirajo različne vrste vzorcev zelo kompleksno.

Na osnovi naših izkušenj smo razvili sistem odločanja za izbor ekstrakcije DNA za posamezen vzorec. V laboratoriju uporabljamo tri metode za ekstrakcijo DNA. Za večino vzorcev uporabimo NucleoSpin® Food, za nekatere vzorce (npr. gluten, lecitin in čokolada) je najbolj primerna izolacija s cetiltrimetilamonijevim bromidom (CTAB), pri ekstrakciji DNA iz listov pa uporabljamo CTAB ali DNeasy Plant Mini Kit. Vzorce smo glede na pridobljene izkušnje o uspešnosti ekstrakcije razdelili na poznane vzorce, ki smo jih že večkrat testirali, in nove vzorce. Poznane vzorce ločujemo na stabilne vzorce, za katere smo pokazali, da iz njih vedno pridobimo DNA ustrezne kvalitete in v zadostni količini, in na variabilne vzorce, pri katerih je lahko učinkovitost ekstrakcije zelo različna zaradi zgoraj omenjenih razlogov. Novi vzorci so tisti, s katerimi še nimamo izkušnje o učinkovitosti ekstrakcije DNA. Skladno s to razdelitvijo smo naredili shemo odločanja, ki je prikazana na Sl. 1. Obenem smo za vzorce z različnimi vsebnostmi posamezne rastlinske vrste (koruze, soje, lanu, krompirja, paradižnika, oljne ogrščice) podali natančne informacije o rezultatih pridobljenih pri nadaljnji analizi DNA z metodo PCR v realnem času.

Vzpostavljeni sistem odločanja izbora metode ekstrakcije DNA je narejen na primeru določanja GSO, vendar je uporaben tudi za druge podobne analize nukleinskih kislin v obravnavanih vrstah vzorcev.

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