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ORIGINAL PAPER

Influence of cocoa components on the PCR detection of soy lecithin DNA

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Abstract The labelling of genetically modified organisms (GMOs) is mandatory in the EU. To comply with this regulation, the International Standardisation Organisation recently published standards for the extraction of DNA and PCR detection of GMOs. The PCR detection of (GM) soy lecithin in chocolate however is not an easy assignment. For the most part this is due to inhibitors such as polyphenols resulting from the cocoa mass and the low level of soy lecithin present. Many factors however, such as the choice of extraction method, sample size and PCR protocol influence the PCR end result. Therefore, preconditions regarding the quality, quantity and purity of the extracted DNA should be carefully determined to guarantee a successful GMO analysis.

Keywords Chocolate · GMO detection · Lecithin · PCR inhibition · ISO

Introduction

Current EU legislation mandates the labelling of genetically modified (GM) food and feed products [1, 2]. Only when the content of genetically modified organisms (GMOs) is below 0.9% no labelling is required, provided that this presence is adventitious or technically unavoidable. This percentage refers to every individual ingredient in a product, e.g. 0.9% GM in the lecithin used in a chocolate bar.

The polymerase chain reaction (PCR) is probably the most widely used technology to check the implementation of these EU regulations. A major limitation of the PCR-based detection of GM-DNA however is the suitability of this DNA with respect to quality, purity and quantity.

The presence of polyphenols, which are powerful antioxidizing agents present in many plants species, can reduce the yield and purity of extracted DNA [3]. Polyphenols can bind covalently to proteins and nucleic acids while in their oxidized forms. This irreversible binding results in a brown gelatinous material that makes the extracted DNA unavailable for most research applications, including restriction enzyme digestion, amplification and cloning [4, 5].

Polyphenols are present in a variety of plants utilized as important components of both human and animal diets [6–8]. Cocoa beans are rich in flavanols. The concentration of flavanols in chocolate depends on both the flavanol content of the cacao plant and the procedures used for transforming the cocoa into chocolate [9, 10]. In a dark chocolate bar of 100 g, total content of polyphenols is 500 mg or higher [11, 12]. Its high content of phenolic compounds makes it difficult to perform DNA based research on chocolate, such as an GMO analysis. In this study, the influence of cocoa mass on the PCR detection of GM soy DNA in self-made chocolate was examined. Focus was put on dark chocolate, as cocoa mass content and therefore plant polyphenols content is higher than in milk chocolate.

Next to the effect of polyphenols, the processing conditions of the food product can affect the detection of DNA, such as exposure to heat, low pH and/or nucleases. In

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chocolate, the low content of soy lecithin, around 0.5% in most cases, can be an additional problem when Roundup Ready® soy should be detected or quantified. Therefore, the lecithin content in different chocolate samples was varied and the PCR detection evaluated. Finally, the influence of the sampling size and DNA extraction method were also evaluated.

Experimental

Preparation of the chocolates

A dark chocolate was prepared with 45.5 g cocoa mass (Barry-Callebaut, Belgium), 10.0 g cocoa butter (Barry-Callebaut, Belgium), 44.0 g sugar (commercial sample) and 0.6 g soy lecithin (Cargill, Belgium), in a total of 100.0 g. The soy lecithin sample was assumed to be 100% GMO. This chocolate will be referred to as the standard chocolate. First, cocoa mass and part of the sugar were poured in a mortar bowl and slowly pre-warmed in a warm water bath at 60–70 °C until the cocoa mass melted, while mixing with a spoon. The mixture was then further homogenised in a mortar. After 15 min, solids were refined using a three-roll refiner. The mixture was then conched in an open warm water bath, set at a temperature of 70 °C, for 4 h. Finally, cocoa butter and lecithin were pre-warmed and added to the conch. The mixture was then further homogenised for 30 min at the same temperature. Chocolate was then transferred to a mould, covered, cooled to room temperature (no tempering was applied) and stored at 4 °C until DNA was extracted.

Isolation of DNA

Nucleon™ PhytoPure™ DNA from plant tissue kit (Amersham Pharmacia Biotech, The Netherlands). DNA was extracted from a 1000 mg sample according to the manufacturer's manual. In the final step, the DNA pellet was washed with 500 μl of a 70% ethanol solution. After vacuum drying, the pellet was eluted in 75 μl bidistilled water.

CTAB method. For extraction with CTAB, the DNA isolation protocol prepared by the European Committee for Standardization (CEN) and published by the International Standardisation Organisation (ISO) was used [13]. The DNA pellet was redissolved into 75 μl bidistilled water.

PVP method. For this method, a sample of 250 mg is used and DNA is extracted according to the protocol of the ISO 21571 standard [13]. In the final step, the DNA pellet was redissolved in 75 μl of bidistilled water.

Wizard® DNA Clean-Up System (Promega, The Netherlands). For an extra purification of the DNA samples, the Wizard DNA Clean-Up System was used. According to the instructor's manual, 50 μl of the DNA extract was used. After clean-up, the samples were eluted in 50 μl bidistilled water.

DNA of 2000 mg lecithin samples was also isolated using a method based on the extraction with hexane [14].

PCR analyses

The primers plant c (5′-CGA AAT CGG TAG ACG CTA CG-3′) and plant d (5′-GGG GAT AGA GGG ACT TGA AC-3′) detect a 500–600 bp fragment of a non-coding region of the chloroplast genome [15]. For amplification, the following PCR mix was composed: 2 μl of extracted DNA product was added to 36.6 μl bidistilled water, 5 μl of 10 × GeneAmp® PCR buffer (Applied Biosystems, Belgium), 2 μl of each primer (10 μM), 2 μl dNTPs solution (5 mM) and 0.4 μl AmpliTaq® DNA polymerase (5 U/μl; Applied Biosystems).

The primers GMO3 (5′-GCC CTC TAC TCC ACC CCC ATC C-3′) and GMO4 (5′-GCC CAT CTG CAA GCC TTT TTG TG-3′) are specific for the detection of the single-copy lectin gene *le1* and yield a PCR product of 118 bp [16, 17]. The lectin gene is present in transgenic as well as in conventional soybeans. For the amplification of the lectin gene, two different PCR set-ups were used. The first set-up (referred to as GMO3/4 method 1) was adapted from the ISO 21569 standard [17] and is similar to the set-up used for the plant c/d PCR. The second set-up (referred to as GMO 3/4 method 2), used 2 μl DNA extract, 26.75 μl of bidistilled water, 5 μl of 10 × PCR buffer (Qiagen, The Netherlands), 10 μl of Q solution (Qiagen), 2 μl of each primer (10 μM), 2 μl dNTPs solution (5 mM) and 0.25 μl of hot-start Taq polymerase (5 U/μl, Qiagen).

For the GMO screening, primers p35S-cf3 (5′-CCA CGT CTT CAA AGC AAG TGG-3′) and p35S-cr4 (5′-TTC TCT CCA AAT GAA ATG AAC TTC C-3′) for the detection of the 35S promoter (123 bp) and primers HA-*nos*118-f (5′-GCA TGA CGT TAT TTA TGA GAT GGG-3′) and HA-*nos*118-r (5′-GAC ACC GCG CGC GAT AAT TTA TCC-3′) for the 3′-*nos* terminator (118 bp) were used [17, 18]. For p35S-CF3/CR4 and HA-*nos*118F/R reactions, 5 μl of a 1/5 dilution of the DNA sample solution was added to a PCR mix, containing 12.04 μl of bidistilled water, 2.5 μl of 10 × GeneAmp® PCR buffer II (Applied Biosystems, Belgium), 1.5 μl of a 25 mM $MgCl_2$ solution (Applied Biosystems), 1.5 μl of primer 1 (10 μM), 1.5 μl of primer 2 (10 μM), 0.8 μl dNTPs solution (5 mM, Promega) and 0.16 μl AmpliTaq Gold™ polymerase (5 U/μl, Applied Biosystems). Both PCR methods are proposed by the CEN and were published recently by the ISO [17].

To detect Roundup Ready® soy, the event specific primer pair *nos*RR2 (5′-GCG CGG TGT CAT CTA TGT TA-3′) and *nos*RR4 (5′-AGG TGT CGC CTT CCT TAC G-3′) was used,

Table 1 PCR thermal profiles applied for the different primer pairs

Primer pair	Plant c/d		GMO3/4 method 1		GMO3/4 method 2		35S-CF3/CR4		HA-*nos*118F/R		*nos*RR2/4	
Initial denaturation	3 min	95 °C	5 min	95 °C	15 min	95 °C	10 min	95 °C	10 min	95 °C	3 min	95 °C
Denaturation	20 s	95 °C	30 s	95 °C	45 s	95 °C	25 s	95 °C	25 s	95 °C	20 s	95 °C
Annealing	40 s	54 °C	30 s	60 °C	45 s	60 °C	30 s	62 °C	30 s	62 °C	40 s	54 °C
Extension	30 s	72 °C	30 s	72 °C	25 s	72 °C	45 s	72 °C	45 s	72 °C	30 s	72 °C
Final extension	3 min	72 °C	3 min	72 °C	5 min	72 °C	7 min	72 °C	7 min	72 °C	3 min	72 °C
Cycles	35		35		41		50		50		35	

resulting in a 177 bp amplicon [19]. The same PCR set-up as for the plant c/d and GMO3/4 (method 1) was used.

Table 1 lists the PCR thermal profiles applied for the different primer pairs.

DNA analysis

DNA fragments were separated by gel electrophoresis in a 2% agarose gel with TAE buffer, stained with ethidium bromide and visualised with a UV transilluminator system. A 100 bp DNA ladder was used to estimate the size length of the DNA fragments. DNA integrity was checked by loading 10 μl of the extracted DNA on an agarose gel. The DNA concentration was determined spectrophotometrically. The proportion of the absorbances at 260 and 280 nm was used as an indicator for the purity of the DNA.

Results

DNA content of chocolate ingredients

The DNA contents of the raw materials were examined in search for absence or presence of (soy) DNA. DNA was extracted from sugar, lecithin, cocoa mass and cocoa butter with the CTAB extraction method and Nucleon Phytopure kit, as these methods have proven to be successful for the extraction of DNA from chocolate samples [20]. The integrity of the DNA extracts was checked through agarose gel electrophoresis.

For the samples extracted with the CTAB method, no DNA smears were observed after agarose gel electrophoresis. After PCR amplification with the universal plant c/d primers, only a positive signal was obtained for the lecithin sample. Spectrophotometrical analysis of the samples revealed concentrations of 10 ng/μl for the lecithin, 44 ng/μl for the cocoa mass and concentrations < 10 ng/μl for the cocoa butter and sugar.

With the Nucleon Phytopure kit, no DNA smears could be visualised for the sugar and cocoa butter samples. However, smears of low molecular DNA fragments (< 600 bp) were obtained for the cocoa mass and lecithin (data not shown). The intensity of the smears from the cocoa mass samples was much higher than for the lecithin samples. For the lecithin, a mean DNA concentration of about 42 ng/μl was obtained, whereas a concentration of 254 ng/μl was measured for cocoa mass. Amplification with the universal plant c/d primers however resulted negative for the cocoa mass and positive for the lecithin. This discrepancy was believed to be due to the presence of PCR inhibitors in the cocoa mass. The A_{260}/A_{280} values, measured via the spectrophotometer, were 1.5 for the cocoa mass and 2.0 for the lecithin. These values confirmed somehow the fact that the cocoa mass DNA extracts were less pure than the lecithins, causing an inhibition of the PCR.

Nevertheless, these results show that the total DNA content can be predominantly attributed to the cocoa mass in first place and to the lecithin in second place. For the cocoa butter, negative results on the agarose gels could be due to hydrolysis of the DNA due to higher pH values during alkalisation of the cocoa mass, removal of DNA from the oil phase during pressing and/or insufficient DNA extracting capacities of the used methods. The absence of DNA sugar is caused by hydrolysis due to alkaline pH and high temperature or nucleolytic degradation [21].

Effect of heat on the DNA stability of soy lecithin

Before any chocolate was manufactured, the influence of the heat treatment during conching on the DNA stability of soy lecithin was examined. The conching environment was mimicked by placing soy lecithin in a warm water bath at 70 °C for 30 min. DNA was extracted [14] from samples before and after the heat treatment and loaded on an agarose gel. Although no significant decrease of the intensity of DNA smears due to the heat treatment could be observed by agarose gel electrophoresis, spectrophotometrical analysis revealed a decrease of the DNA content by 5–40%. Nevertheless, this decrease had no effect on the PCR amplification of these samples. For the detection of the non-coding region of the chloroplast genome with primer pair plant c/d, as well as for the detection of the soy lectin gene with primer pair GMO3/GMO4 (PCR method 1), clear positive signals were observed before and after the heat treatment of the lecithin. Taking these results into account, it was assumed

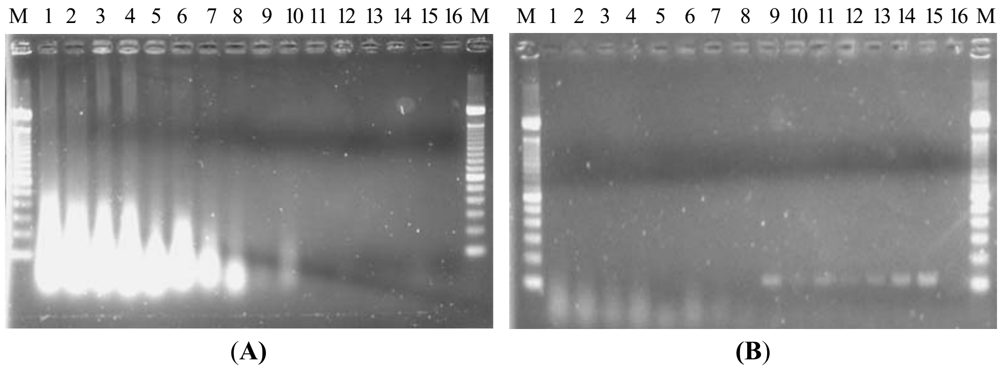


Fig. 1 Evaluation of DNA from chocolate with different amounts of cocoa mass, extracted with the Nucleon Phytopure kit, through (**A**) agarose gel electrophoresis and (**B**) amplification of soy DNA with PCR method 2. *M:* 100 bp DNA length marker, *lanes 1* and *2*: chocolate with 55.4% cocoa mass (CM) and 0.0% cocoa butter (CB), *lanes 3* and *4*: 45.4% CM and 10.0% CB, *lanes 5* and *6*: 35.4% CB and 20.0% CB, *lanes 7* and *8*: 25.4% CM and 30.0% CB, *lanes 9* and *10*: 15.4% CM and 40.0% CB, *lanes 11* and *12*: 5.4% CM and 50.0% CB, *lanes 13* and *14:* 0.0% CM and 55.4% CB, (**A**) *lanes 15* and *16:* negative extraction control, (**B**) *lane 15:* positive PCR control, *lane 16:* negative PCR control

that soy lecithin DNA is quite stable during the conching of chocolate.

Influence of the cocoa mass content on PCR detection of soy lecithin

The influence of the cocoa mass content on the DNA extraction and PCR amplification was examined. Chocolates were made with different amounts of cocoa mass (CM), varying from 55.4 to 0.0%. The amount was exchanged with the cocoa butter (CB) added, while sugar and lecithin content remained constant, at 44.0 and 0.6%, respectively. DNA was extracted with the CTAB method and the Nucleon Phytopure method.

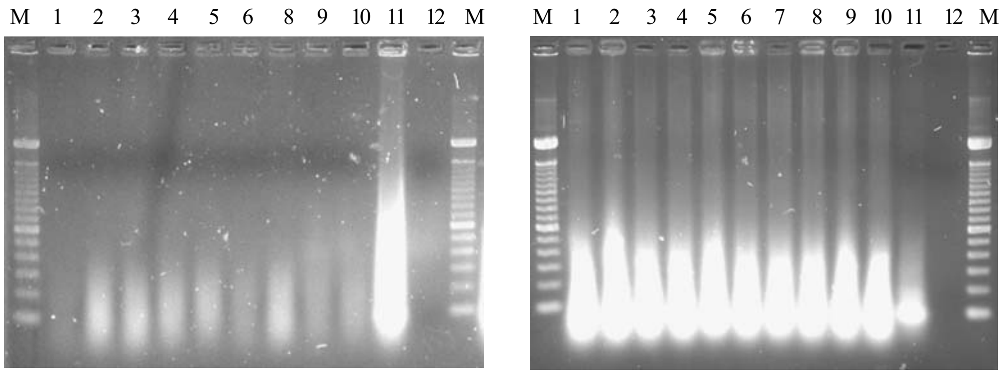
Clearly more DNA could be extracted with the Nucleon Phytopure kit for all samples than with the CTAB method. Spectrophotometrical analysis revealed a mean concentration of 2501 ng/μl for the standard chocolate extracted with the kit, whereas for the pure cocoa mass sample a mean concentration of 254 ng/μl was obtained. These results suggest that the DNA extraction is strongly inhibited by the cocoa mass.

Analysis of the DNA integrity of the chocolate samples on an agarose gel showed a decreasing intensity of the smears as less cocoa mass was added to the sample (Fig. 1A). Obviously, the cocoa mass is the major component contributing to the DNA content of the samples. After amplification of the lectin gene of these samples with PCR method 2, only a positive amplification was obtained for samples with a cocoa mass content of 15.4% or lower (Fig. 1B). Although DNA could be isolated in rather high contents for the samples with 25.4% cocoa mass or higher, no amplification could be obtained for these samples. Clearly, the presence of the cocoa mass exerts an inhibitory effect on the soy DNA amplification.

For the chocolate samples extracted with the CTAB method, similar results were obtained compared to the Nucleon phytopure kit. However, less DNA could be extracted. Detection of the soy lectin gene with GMO3/4 primers, using PCR method 1, resulted negative for all samples, even for the sample where no cocoa mass was present. PCR using method 2 resulted in clear positive signals for the chocolate with 25.4 and 15.4% cocoa mass (data not shown).

Low A_{260}/A_{280} values were obtained for all samples. Generally, values for the CTAB method were lower than for the Nucleon Phytopure method (<1.11 and <1.46, respectively). A small tendency for decreasing A_{260}/A_{280} values with decreasing cocoa mass content could be noticed. In other words, the inhibiting effect of the cocoa mass is not translated in the A_{260}/A_{280} values of the DNA extracts.

Further PCR analysis revealed that for the screening of GMOs, with the detection of the 35S promoter and the 3′-*nos* terminator, negative results were obtained for all samples extracted with the CTAB method. An extra clean-up of the samples with the Wizard® DNA Clean-Up System did not improve these results. For the samples extracted with the Nucleon Phytopure kit, positive results were only obtained for the samples were no cocoa mass was added. The inhibiting effect of the cocoa mass is therefore very much present in this PCR set-up. The cleaning up of the samples however strongly reduced the presence of the PCR inhibitors. For most of the samples, a positive result was obtained, although these results could not always be reproduced. Apparently, some influence of the cocoa mass inhibitors was still present. The PCR detection of the Roundup Ready® soy specific junction region with *nos*RR2/4 primers resulted negative for all samples, independent of the extraction method used.



(**A**) (**B**)

Fig. 2 Evaluation of DNA from chocolate with different amounts of soy lecithin extracted with (**A**) a PVP-based method, and (**B**) the Nucleon Phytopure method, through agarose gel electrophoresis. *M:* 100 bp DNA length marker, *lanes 1* and *2*: chocolate with 0.6% lecithin, *lanes 3* and *4*: 0.8% lecithin, *lanes 5* and *6*: 1.0% lecithin, *lanes 7* and *8*: 2.0% lecithin, *lanes 9* and *10*: 3.0% lecithin, *lane 11*: positive extraction control, *lane 12*: negative extraction control

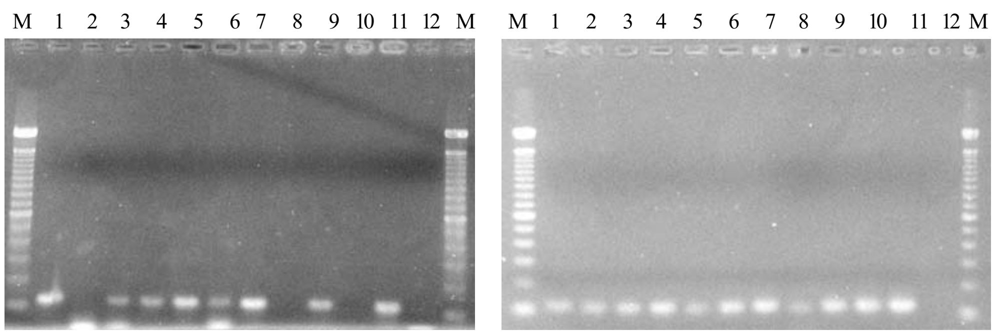
Influence of the amount of soy lecithin on PCR detection

In order to determine the amount of soy lecithin which should be present in chocolate to obtain a positive amplification result using the traditional PCR method as described in the ISO 21569 standard, i.e. with the GMO3/4 method 1, chocolates were made with different amounts of lecithin, ranging from 0.6 to 3.0%. These amounts were corrected with the amount of cocoa butter added to the chocolate. DNA was extracted with the CTAB method, a PVP based method and the Nucleon Phytopure kit.

Results of the DNA extracts loaded on an agarose gel are shown in Fig. 2A for the PVP method and in Fig. 2B for the Nucleon Phytopure kit. With the CTAB method, very light DNA smears could be visualised also, with a length ranging from 100 to 800 bp (data not shown). Among the DNA extraction methods applied, the Nucleon Phytopure kit performed better at recovering high concentrations of in particular short DNA fragments (< 600 bp) (Fig. 2B).

PCR results were poor: positive amplification results could be obtained for the PVP and Nucleon Phytopure samples with a lecithin content of 1% lecithin and higher, after an extra clean-up (data not shown). However, results could not always be reproduced. Most probably, the difficult detection is due to the combined effect of PCR inhibitors and a low amount of target DNA.

For all chocolate samples, a lecithin sample was used with a presumable GMO content of 100%. Screening for GMO should therefore give positive results. For all samples, i.e. samples extracted with the CTAB method, the Nucleon Phyotpure kit and the PVP method, a screening for the 35S promoter and the 3′-*nos* terminator was performed. PCR results however were irreproducible for the samples extracted with the CTAB and PVP method, due to the presence of residual PCR inhibitors (Fig. 3A). This could be explained by the fact that both methods are based on the precipitation



(**A**) (**B**)

Fig. 3 Amplification of the 35S promoter from chocolate with different amounts of soy lecithin extracted with (**A**) a PVP-based method and (**B**) the Nucleon Phytopure kit, after clean-up. *M:* 100 bp DNA length marker, *lanes 1* and *2*: chocolate with 0.6% lecithin, *lanes 3* and *4*: 0.8% lecithin, *lanes 5* and *6*: 1.0% lecithin, *lanes 7* and *8*: 2.0% lecithin, *lanes 9* and *10*: 3.0% lecithin, *lane 11*: positive PCR control, *lane 12*: negative PCR control

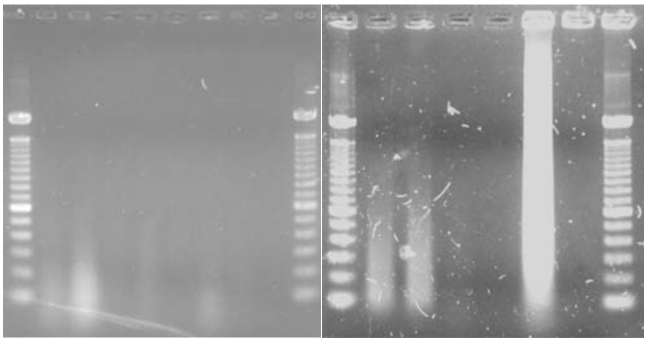


Fig. 4 Evaluation of DNA isolated with the CTAB method from chocolate using different sample sizes through agarose gel electrophoresis. *M:* 100 bp DNA length marker, *lanes 1* and *2*: 5.0 g sample, *lanes 3* and *4*: 10.0 g sample, *lanes 5* and *6*: 15.0 g sample, *lanes 7* and *8*: 20.0 g sample, *lanes 9* and *10:* pooling 10 × DNA from 1.0 g samples, *lanes 11* and *12:* extracting 1 × 10.0 g sample, *lane 13*: positive extraction control, *lane 14*: negative extraction control

of DNA in the test tube. As a result, some DNA might get lost in some cases during e.g. washing procedures. Only the Nucleon Phytopure kit gave positive results for all samples from 0.6 to 3.0% lecithin (Fig. 3B). These results were reproducible and intensity of the amplification bands were similar for all samples, indicating that for this extraction procedure the effect of the PCR inhibitors is reduced to a minimum. The Roundup Ready® soy line specific detection resulted negative for all samples of all extraction methods tested (data not shown).

Influence of sample size on PCR detection of soy lecithin

For the extraction of DNA from chocolate with the CTAB method, an increase of the sample size from 300 to 1000 mg resulted in much better results. In the latter case, 2 ml of extraction buffer was used. Especially in cases where the extraction method is not based on the use of spin columns, scaling up to larger sample volumes is quite simple. Nevertheless, it was still not possible to detect the soy lecithin DNA present in the samples through the 35-cycle PCR program proposed by the CEN. Therefore, DNA was extracted from chocolate samples up to 20.0 g with the CTAB method. The results show that the higher sample size does not necessarily result in higher DNA yields, as shown in Fig. 4, lanes 1–8. For the 5.0 g sample, a clear smear of low molecular weight DNA could be observed, whereas for the samples with higher sample size, the intensity of the smear was lower or no smear could be visualised at all. This could be explained by the fact that proportions of sample size to buffer volumes were changed, due to the limited size of the reaction tubes. As a result, the efficiency of the DNA extraction decreased. Moreover, in the case of chocolate, higher amounts of the sample also means higher amounts of DNA extraction and PCR inhibitors.

These results were confirmed through PCR analysis: higher sample sizes were not the key to obtain (more) positive amplification results. Neither with the plant c/d nor the GMO3/GMO4 primers (method 1) PCR products were obtained. Very light amplification signals could be obtained with the 41-cycle PCR program (method 2) for the 15.0 and 20.0 g samples, but results were not reproducible.

To avoid the problem of changes in proportions of sample to buffers, DNA was pooled. DNA was obtained from 10 independent extractions of 1.0 g chocolate and compared with DNA extracted from 10.0 g sample. For the pooled DNA, the 10 pellets were gathered at the point in the extraction procedure where they are washed with ethanol.

Spectrophotometrical analysis as well as agarose gel electrophoresis revealed higher yield for the pooled DNA. Pooling the DNA resulted in DNA concentrations of about 345 ng/μl, while for the 10.0 g sample, similar values as before were obtained (ca. 48 ng/μl). The A_{260}/A_{280} purity factor did not significantly differ. Analysis on the agarose gel clearly showed the difference between the two procedures, as shown in Fig. 4, lanes 9–14. For the pooled samples, a clear DNA smear could be observed, but only a very light or no signal could be seen for the 10.0 g sample.

Results of the PCR analysis of the samples were in agreement with the previous results. That is, for the DNA extracts after clean-up. Without a clean-up of the samples, no DNA could be amplified, probably due to the high content of PCR inhibitors. A clean-up resulted in purer samples (higher A_{260}/A_{280} values, ca. 1.29, while 1.16 without clean-up), but reduced the DNA content significantly for the pooled sample. The concentration of 345 ng/μl was reduced to 37 ng/μl, whereas for the 10.0 g sample, the concentration remained the same (ca. 51 ng/μl). Amplification with the plant c/d primers gave clear positive results for the pooled DNA, as negative results were obtained for the 10.0 g sample (data not shown). Although DNA is lost during the cleaning up of the samples, still some DNA is left and PCR inhibitors are significantly reduced enabling PCR analysis. The same results were obtained when performing a PCR with GMO3/GMO4 primers, using method 2. Still, no DNA could be amplified with method 1.

The detection of the 35S promoter and the 3′-*nos* terminator also resulted negative for the 10 g and the pooled sample, nor could any positive amplification result be obtained after amplification with *nos*RR2/RR4 primers.

Discussion

Results clearly show that the PCR detection of genetically modified soy lecithin from chocolate is not an easy assignment. In this research, several DNA extraction methods were compared, two of which form part of an international ISO standard [17]. The comparison of methods intended to demonstrate to a maximum the inhibitory effect of the cocoa mass. After all, when used in routine GMO analysis, a DNA extraction method should be chosen with a good performance for a wide range of products. It is not workable to perform many different extraction methods for every different type of sample.

All methods showed that the presence of cocoa mass, and likely in particular the high levels of polyphenols, hinder an efficient DNA extraction and PCR analysis. This is especially true for dark chocolate, where the amount of cocoa mass is higher compared to white and milk chocolate. Due to the presence of inhibitors, the evaluation of the extracted DNA through agarose gel electrophoresis appears to be not fully reliable in predicting PCR results. In some cases, no smear can result in positive amplification, whereas the opposite has also been shown. Neither can the A_{260}/A_{280} value, reflecting the purity of the DNA extract, be relied on. This is most probably because it predicts for presence of protein (low value, < 1.7) or RNA (high value, > 2.0). As a result, it cannot be solely used as a predictor for the success of a subsequent PCR.

Difficult GMO detection however is not necessarily or only due to matrix effects, but could also be due to the low level of target DNA. In the case of chocolate, an amount of 0.6% soy lecithin appears to render the PCR amplification extra difficult. Amounts of 1 and 2% soy lecithin or higher are needed to enable positive amplification for the lectin gene, using the 35 PCR-cycles method proposed by the CEN. Such high amounts are however not used in chocolate manufacturing.

The GMO screening PCRs used in this research have been validated on a European level [18] and form part of the ISO 21569 standard [17]. Together with the previous results, it is shown that these PCRs might result positive in cases where the amplification of the lectin gene remains unfeasible. The species specific PCR is less sensitive than the GMO screening PCR. The same goes for the line specific detection of Roundup Ready® soy. This can be easily explained by the difference in amplification cycles: GMO screening PCRs involve 50 cycles, while species and GMO specific PCR involve only 35 cycles. To solve this discrepancy, two possibilities emerge: increase the sensitivity of the species and GMO specific PCRs, or decrease the sensitivity of the GMO screening PCRs. From an analytical point of view, the first option would be most appropriate. Nevertheless, an increase of the amount of PCR cycles increases the risk for false-positive results.

Conclusions

The detection of soy lecithin in chocolate is not an easy assignment. Although heat treatment during conching does not strongly affect the DNA, PCR detection is difficult due to the presence of cocoa mass, which strongly inhibits DNA extraction and PCR analysis. The content of target DNA, in this cases originating from the lecithin, is also quite low. An increase of the sampling size has shown to increase DNA yields, but this can not be done infinitely, due to practical considerations and changes in the extraction efficiency.

The choice of an extraction method very much influences the PCR results obtained. For a laboratory conducting GMO analyses routinely, an extraction method with a wide application range may be favoured, but the possible limitations of such a method should always be kept in mind. Although ISO international standards have been published, these methods do not guarantee the best results for the product under investigation. Therefore, future research should be focussed on the determination of the preconditions for a successful GMO analysis, including quality, quantity and purity of the DNA, in relation to specific matrices. For this purpose, specific analytical tools should be developed and/or optimized.

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