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Detection of processed genetically modified food using CIM monolithic columns for DNA isolation

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Abstract

The availability of sufficient quantities of DNA of adequate quality is crucial in polymerase chain reaction (PCR)-based methods for genetically modified food detection. In this work, the suitability of anion-exchange CIM (Convective Interaction Media; BIA Separations, Ljubljana, Slovenia) monolithic columns for isolation of DNA from food was studied. Maize and its derivates corn meal and thermally pretreated corn meal were chosen as model food. Two commercially available CIM disk columns were tested: DEAE (diethylaminoethyl) and QA (quaternary amine). Preliminary separations were performed with standard solution of salmon DNA at different pH values and different NaCl concentrations in mobile phase. DEAE groups and pH 8 were chosen for further isolations of DNA from a complex matrix—food extract. The quality and quantity of isolated DNA were tested on agarose gel electrophoresis, with UV-scanning spectrophotometry, and by amplification with real-time PCR. DNA isolated in this way was of suitable quality for further PCR analyses. The described method is also applicable for DNA isolation from processed foods with decreased DNA content. Furthermore, it is more effective and less time-consuming in comparison with the existing proposed methods for isolation of DNA from plant-derived foods.

Keywords: DNA isolation; Monolithic columns; Convective interaction media; Food control; Processed foods; Genetically engineered food; Genetically modified food; Genetically modified organisms; Maize; Polymerase chain reaction; Real-time polymerase chain reaction

1. Introduction

The availability of sufficient quantities of pure and intact DNA is always a crucial point in DNA-based methods, i.e. for polymerase chain reaction (PCR), DNA sequencing, Southern blotting, and microarrays [1]. The same is true for the DNA-based methods for detection of genetically modified (GM) food [2]. According to the European legislation, food products must be labeled as genetically modified, if GM compounds are present in proportion higher than 0.9%. GM food means food containing, consisting of or produced from genetically modified organisms (GMO) [3,4]. The majority of the existing methods for the detection of GM food is based on testing for the presence of recombinant DNA, or on the detection of novel expressed proteins [5]. Near-infrared (NIR) spectroscopy is also applicable, if there are enough differences between the conventional and the engineered food [6]. DNA-based methods include Southern blotting, conventional qualitative PCR, quantitative competitive PCR, and qualitative or quantitative real-time PCR [5].

During the production chain, food passes several physical, biological, and chemical processes, which mostly negatively influence the quantity and quality of available DNA [7,8]. Three different approaches for DNA isolation from plant material and plant-derived products are the most widely used for GM food detection: the cetyltrimethylammonium bromide (CTAB) method [9], DNA-binding silica columns in

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form of various commercial kits and a combination of both. The existing methods for DNA isolation from food cannot always fulfill the expectations regarding quantity and quality of isolated DNA. Furthermore, they usually require only up to 100 mg of food sample and are difficult to scale-up [10].

Chromatographic methods were traditionally used for the isolation of proteins from various sources, such as microbial culture, animal tissue and plants. More recently, with emerging techniques in biotechnology and bioengineering, the isolation of recombinant proteins, peptides, carbohydrates and nucleic acids has gained importance [11]. The supports for stationary phases applied in chromatography of large biomolecules must fulfill some distinct criteria, different from those for the chromatography of low-molecular-mass biologically non-active molecules. The major problem is the restricted access of the biomolecules to the small pores of the classical porous chromatographic supports. The so-called wide-pore silica columns opened a broad field of new applications in the chromatography of biomolecules [12]. Some problems such as the slow mass transport resulting in peak broadening and low recovery still remained unsolved. Another approach, which allowed overcoming the problem of mass transport, is the application of micropellicular supports characterized by a spherical fluid-impervious core of support material, covered by a thin retentive layer of stationary phase. Because of the lack of pores, the surface area is low, but rapidly and efficiently available for the mobile phase and the sample molecules [1,13]. In the late 1980s, a novel type of supports has been introduced, called monoliths [14]. Monoliths are constituted of a single piece of continuous and highly porous material, which is arranged in a way to form a network of highly interconnected channels. In such media, the mobile phase is forced to flow trough the pores. As a consequence, the mass transport is enhanced by convection, what contribute to minimize the void volume and the peak broadening [15,16].

Five major chromatographic modes are used for the separation of nucleic acids: size-exclusion [17], anion-exchange [11], mixed-mode [18], ion-pair reversed-phase [19] and slalom chromatography [20]. Among theme anion-exchange chromatography combined with micropellicular supports, is described as the most prominent technique so far [1]. At neutral pH, the hydrophobic organic bases of the double-stranded DNA molecule are inside, whereas the two sugar-phosphate chains spiral down the outside of the double-helix structure. The sugar-phosphate chains create a poly-anionic, highly hydrated, and hydrophilic surface to the solvent allowing the chromatographic separation in anion-exchange mode. CIM (Convective Interaction Media; BIA Separations, Ljubljana, Slovenia) monolithic columns allow fast and flow-unaffected separation of several biomolecules, including nucleic acids [16]. Anion-exchange chromatography with CIM disks allows the separation of oligonucleotides of different size [21,22] and also the isolation of plasmid DNA from a complex matrix, i.e. *E. coli* cell lysate [23,24]. Bacterial genomic DNA of size up to 200 kbp can be also separated on CIM monoliths [25].

The aim of this study was to set up a fast and effective HPLC method with CIM anion-exchange monolithic columns for the isolation of DNA from maize and its derivates corn meal and thermally pre-treated corn meal with reduced DNA content. The main criteria to be fulfilled were the appropriate quantity and purity of isolated DNA for further PCR analyses. The effects of the mobile phase composition, sample dilution media, pH, and column type on the characteristics of salmon DNA isolation from standard solution were evaluated. Afterwards, CIM DEAE disks were applied for the isolation of DNA from a complex matrix—food extract. The quality and quantity of isolated DNA was checked on agarose gel electrophoresis, with UV-scanning spectrophotometry, and by amplification with real-time PCR.

2. Experimental

2.1. Separation unit and equipment

Isolations of DNA were performed on commercially available CIM DEAE (diethylaminoethyl) or QA (quaternary amine) monolithic columns (BIA Separations). A gradient HPLC system (Knauer, Berlin, Germany) was built of two pumps, a dynamic mixing chamber, an injector with 20, 100, 200 or 1000 μl loop, a variable-wavelength detector and an interface box connected to a computer with EuroChrom 2000 software. Injections of volumes larger than 1000 μl were achieved by multiple injections. The applied columns were in form of a disk, placed in the appropriate housing and integrated in a HPLC system. CIM disks had the diameter of 12 mm and the thickness of 3 mm, what brings 340 μl of column volume. The monitor wavelength was 260 nm and the flow rate was 1 ml/min in all the separations.

2.2. Mobile phase

Fifty millimolar Tris buffer (Merck, Darmstadt, Germany) with pH values from 7 to 12 was used as mobile phase. The pH value was adjusted with 1 M NaOH (Merck) or with 1 M HCl (Merck). A 3.5 min linear gradient of increasing salt concentration was applied, including 2 M NaCl in the elution buffer and varying values in the loading buffer.

2.3. DNA samples for chromatographic isolations

Aqueous solution of double-stranded DNA (deoxyribonucleic acid sodium salt from salmon testes, Sigma–Aldrich, Taufkirchen, Germany, catalog No. ID1626) and its solution in chromatographic buffer were used as internal standards for the initial testing of the HPLC system.

2.4. Food samples

Corn meal and thermally pre-treated corn meal for fast preparation of the traditional Mediterranean dish “po-

lenta" were chosen as model foods for the DNA isolations. Thermally pre-treated corn meal was prepared industrially from corn meal by treating with steam at high pressure (3.3–3.4 bar) and subsequently dried with hot air (190–195 °C). A sample of raw maize with traces of GMO line MON810 under 0.5% was kindly provided by the Austrian Institute for Seed (Agricultural Inspection Service and Research Institute Vienna, Federal Office for Food Safety). The samples were homogenized with a coffee grinder.

2.5. Food extract preparation for chromatographic isolations

The extract from food samples was prepared as following: 3 ml of sterile double distilled water and 5 ml of CTAB buffer (20 g/l CTAB, 1.4 M NaCl, 0.1 M Tris–HCl, 20 mM EDTA, pH 8) were added to 1 g of a homogenized sample. The sample was then incubated at 65 °C for 0.5 h, mixed three times during incubation and centrifuged at 4000 rpm for 10 min. Because of the high salt concentration, it was diluted before the isolation with sterile double distilled water at the ratio of 3:4. Therefore, each 100 μl of diluted extract contained DNA from 10 mg of food sample. The extract was filtered through a filter of regenerated cellulose with pores of 0.45 μm before the isolation.

2.6. DNA recovery from the HPLC fraction

The 700 μl fractions with purified DNA were collected and DNA was subsequently recuperated by ethanol precipitation as following: addition of 2 volumes of cold absolute ethanol, incubation on ice for 30 min, centrifugation for 10 min at 13,000 rpm, discard of supernatant, washing with 700 μl of 70% ethanol, centrifugation for 10 min at 13,000 rpm, dried and suspended in 100 μl of TE buffer (10 mM Tris and 1 mM EDTA).

2.7. Agarose gel electrophoresis

The analyses were performed on a Pharmacia Biotech LKB GNA 100 electrophoresis unit, using following conditions: TAE as running buffer [40 mM Tris (Merck), 20 mM acetic acid glacial (Merck), 2 mM EDTA (Kemika, Zagreb, Croatia)], 9 μl of sample and 1 μl of loading buffer (Promega, Mannheim, Germany), 1.5% agarose (medium EEO, Sigma, Taufkirchen, Germany), 120 V, 50 min. The gel was subsequently evaluated by ethidium bromide staining (0.5 μg/ml EtBr).

2.8. UV-scanning spectrophotometry

The measurements of the UV absorbance spectra (220–320 nm) were performed on a Pharmacia Biotech Ultraspec 2000 UV–vis spectrophotometer, with 10× diluted DNA samples in 0.5 ml quartz cuvettes. DNA was dissolved with double distilled water.

2.9. Real-time PCR analyses

Reactions were performed on an ABI PRISM 7900HT instrument (Applied Biosystems, Foster City, CA, USA) with 20 μl reaction mixture containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems) and using the following cycling conditions: 2 min at 50 °C, 10 min at 95 °C, 50 cycles of 15 s at 95 °C, and 1 min at 60 °C. The primers and probe specific for maize invertase gene [26] were used to determine the amount of maize DNA in food samples. The presence of GM maize MON810 in the samples was confirmed using the GM line specific primers and probe [27]. The concentration of the primers for the invertase gene was 900 nM and of the probe was 200 nM. For the MON810 specific assay, the concentration of primers was 600 nM and of the probe was 150 nM. Known amounts of DNA from the certified reference material (5% GM maize MON810, Institute for Reference Materials and Measurements, Belgium, catalog No. BF413c) were used to prepare the standard curve for the determination of maize DNA and GM maize DNA content. Serial dilutions containing 100, 20, 5, 1 and 0.5 ng of DNA were run on real-time PCR in triplicate. Threshold cycle (C_t) values were determined after manual adjustment of the baseline and the fluorescence threshold line, using SDS 2.1 software (Applied Biosystems). The C_t values of each real-time PCR were plotted against the logarithm of the estimated DNA quantity in the sample. The quantification of DNA amount for unknown samples was performed by interpolation of C_t values generated in the standard regression curve. Standard curves were also plotted for the DNA isolated from food samples and the slope was used for efficiency calculation following the equation: $E = 10^{-[1/\text{slope}]} - 1$, where efficiency of 1 corresponded to 100% PCR efficiency [28,29].

2.10. Additional methods for DNA isolation

Two additional methods were used for isolation of DNA from food samples and for the comparison with the proposed chromatographic method: the CTAB method, as described by Lipp et al. [9], and the DNeasy Plant Mini Kit (Qiagen, Valencia, USA). DNA from the certified reference material for real-time PCR (5% GM maize MON810) was isolated with the GENE*Spin* DNA Extraction Kit (GeneScan, Germany) and quantified with the PicoGreen double-stranded (ds) DNA Quantification Kit (Molecular Probes, Eugene, USA).

3. Results and discussion

Among the applicable methods for the detection of GM food, the PCR method is still the most widely used in research and inspection laboratories [9]. The DNA isolation method is crucial for GM food detection since sufficient amount of DNA is needed for reliable quantification and the impurities can act as inhibitors of the PCR. Usually, the quantity of DNA in a PCR reaction is 100 ng, therefore the concentration of a

5 μl DNA sample must be at least 20 ng/μl. During the production chain, food passes several physical, biological, and chemical processes, which mostly negatively influence the quantity and quality of the DNA available for the isolation. The phenomenon is especially expressive when high temperature treatment is performed at low pH. In spite of the many existing methods for DNA isolation from food, the problem still persists for certain food matrices [7,8].

The double-stranded DNA is a large polymeric and superficially negatively charged molecule and therefore it can be isolated in anion-exchange mode [1,11,22,25]. Because of the distinctive structure with high density of negatively charged phosphodiester groups, it was expected that separation would be significantly affected by the mobile phase composition. To obtain the optimal conditions for DNA isolation from larger volume and from food samples, the effect of pH and NaCl concentration in the loading buffer on the characteristics of separation and on the recovery during gradient separation of DNA was tested. The injected sample was a standard aqueous solution of salmon genomic DNA of size distribution greater than 10 kbp. The recovery was calculated by dividing the sample peak area under binding conditions and the sample peak area without the column. Two commercially available CIM disks were tested: DEAE in the pH range 7–9 and QA in the pH range 10–12.

Small quantities of displacer ions in the loading buffer act as competitors for the binding sites, resulting in a weaker retention and higher recovery. The phenomenon is especially pronounced in the case of DNA molecules, because of their size and very high number of negatively charged binding sites [25]. The same was expected in our experiment. The pH value in the tested area was not expected to influence the charge of DNA molecule. However, the pH value influences the charge of the active groups of the ion-exchanger, thus affecting the separation characteristics [25]. In the case of DEAE groups, increase of the pH with the lowest NaCl concentration had a slightly positive effect on the DNA recovery. In all other cases, there were no significant influences of the pH to the recovery that remained below 70% (Fig. 1). Only higher peak

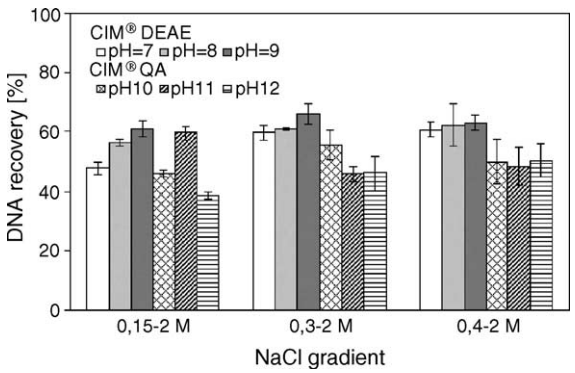

Fig. 1. The effect of pH and NaCl concentration in mobile phase on DNA recovery during isolation with CIM DEAE and QA disks (standard aqueous solution of salmon DNA; sample volume 20 μl; flow rate 1 ml/min; $n \geq 3$).

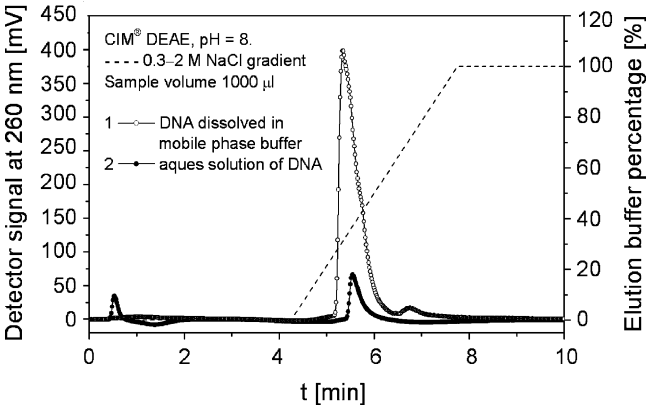

Fig. 2. The effect of sample dilution media on DNA isolation with CIM DEAE disks (1: DNA dissolved in mobile phase buffer, concentration 9.6 ng/μl; 2: aqueous solution of DNA, concentration 6.9 ng/μl; flow rate 1 ml/min).

broadening was perceivable after increasing the pH value. When applying NaCl concentration of 0.6 M, the retention was significantly reduced and nearly all the sample passed through the column without retention. DEAE columns, applying pH 8 and NaCl gradient 0.3–2 M, were chosen for further separations from larger volumes.

To simulate as much as possible real samples, the effect of dilution media and 1000 μl sample injection was investigated. DNA was dissolved in distilled water and in the loading buffer. In Fig. 2, the chromatograms of DNA isolation from differently prepared samples are presented. In contrast to the precedent experiments, where 20 μl samples were injected, in this case samples up to 1000 μl were loaded, that is almost three times the column volume. We can observe that the sample preparation had a considerable effect on the retention. The small peak at 0.5 min was the consequence of the significant amount of injected aqueous DNA sample into the mobile phase buffer and did not comprise any DNA. When the dissolving media of the injected DNA sample and the mobile phase buffer were the same, the peak was not observed. The peak area did not increase proportionally to the quantity of injected sample. Furthermore, the values of recovery decreased with larger injection volumes (Fig. 3). Recovery for the sample dissolved in loading buffer was almost 80%, compared to only 15% for the aqueous solution of DNA. In the case of loading large samples of volume comparable to or even higher than the column volume, the composition became very important in the sense of forming the environment within the ion-exchanger. When the aqueous sample entered the column, water was the main media that formed the surrounding for ion-exchange, in spite of 0.3 M NaCl in mobile phase. As a consequence, there were no competitor ions and the DNA molecules were strongly retained. Further displacement of the molecules was more difficult to achieve, resulting in poor recovery.

Corn meal and thermally pre-treated corn meal were used as model foods for DNA isolations. In contrast to the raw

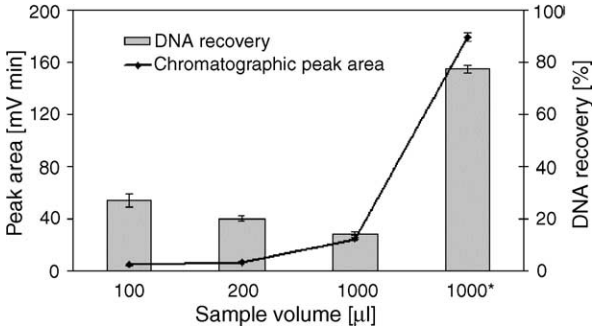

Fig. 3. The effect of sample dilution media on peak area and DNA recovery during isolation with CIM DEAE disks (100, 200 and 1000: aqueous solution of DNA, concentration 6.9 ng/μl; 1000*: DNA dissolved in mobile phase buffer, concentration 9.6 ng/μl; flow rate 1 ml/min, pH 8, linear NaCl gradient 0.3–2 M; $n \geq 3$).

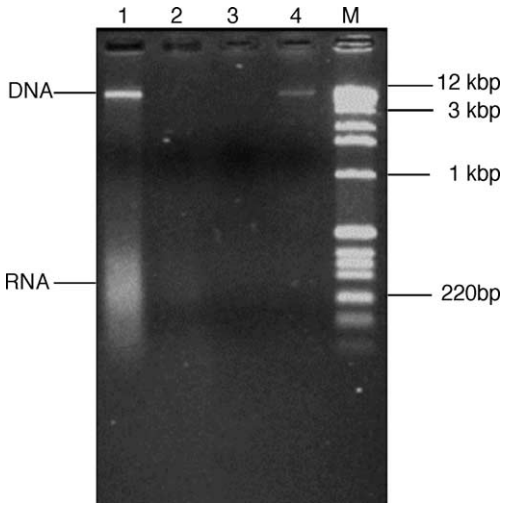

Fig. 4. Agarose gel electrophoresis of DNA isolated from 100 mg of corn meal and thermally pre-treated corn meal (DNA isolation according to the CTAB method: (lane 1) corn meal and (lane 2) thermally pre-treated corn meal; DNA isolation with Qiagen DNeasy Plant Mini Kit: (lane 3) thermally pre-treated corn meal and (lane 4) corn meal; (M) 1 kbp ladder).

sample (Fig. 4, lanes 1 and 4), in case of isolating DNA from the thermally pre-treated sample, DNA was not visible on the electrophoresis (Fig. 4, lanes 2 and 3). DNA was isolated according to the CTAB method [9] and with the DNeasy Plant Mini Kit, which are described as adequate and recommended for the purpose of GM food detection [10,30].

In Fig. 5, the chromatograms of DNA isolation from different quantities of heat pre-treated corn meal extract are presented. The samples were diluted before the isolation with double distilled water at the ratio of 3:4, because of the high salt concentration in the extraction buffer. Extraction buffer with lower salt concentration was tested as well, but the quantities of recovered DNA were lower (data not shown). Also different NaCl gradient were tested and no significant differences were found (data not shown). CIM DEAE disks and mobile phase with pH 8 and linear NaCl gradient of 0.15–2 M NaCl were used. Volumes larger than 1 ml were loaded by

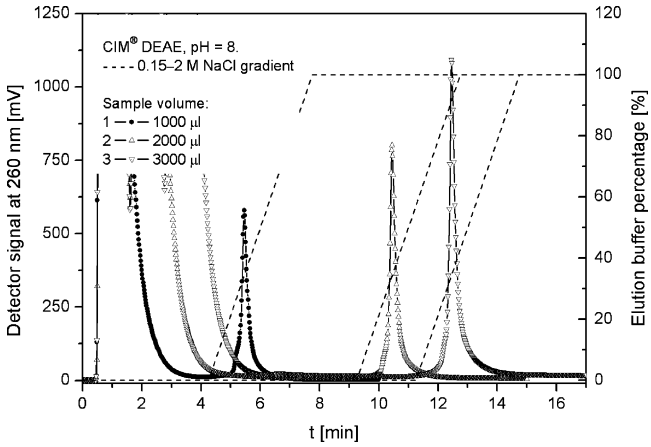

Fig. 5. Chromatograms of DNA isolation with CIM DEAE disks from different quantities of thermally pre-treated corn meal (flow rate 1 ml/min; 1000 μl is correspondent to 100 mg of food sample; peak area: (1) 178.8 ± 2.9, (2) 247.0 ± 10.8, (3) 346.5 ± 10.8; $n \geq 3$).

multiple injections with the 1 ml sample loop. In the case of larger volumes, the loading time was extended, allowing to all the not-binding impurities to pass through the column, before the gradient elution. Because of the mentioned reasons, the retention times for different sample volumes differed. The separation was effective and the peaks were sharp. Separation occurred in a few minutes, during the 3.5 min NaCl gradient. The peak area was proportional to the quantity of loaded sample. The displacer NaCl concentration was slightly below 0.8 M in all the cases.

DNA isolated with CIM DEAE disks were checked on the agarose gel electrophoresis. The DNA from the 200 and 300 mg samples were clearly visible (Fig. 6, lanes 5 and 6)

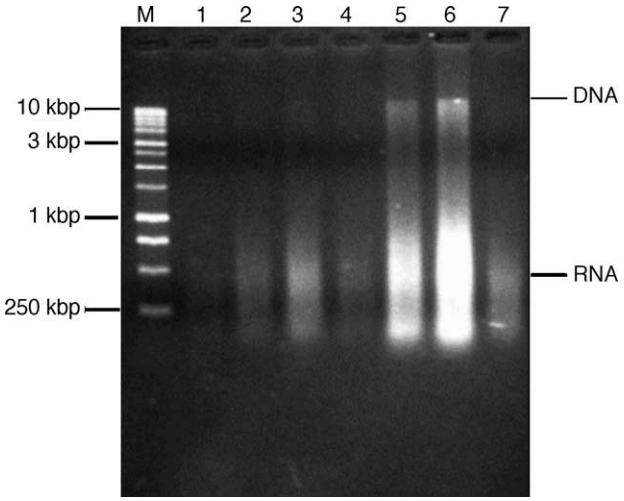

Fig. 6. Agarose gel electrophoresis of DNA isolated according to the CTAB method and with CIM DEAE disks from different quantities of thermally pre-treated corn meal (DNA isolation with CIM DEAE disks from: (lane 1) 10 mg, (lane 2) 20 mg, (lane 3) 50 mg, (lane 4) 100 mg, (lane 5) 200 mg, (lane 6) 300 mg; flow rate 1 ml/min, linear NaCl gradient 0.15–2 M, pH 8; (lane 7) DNA isolation according to the CTAB-method from 100 mg; (M) 1 kb ladder).

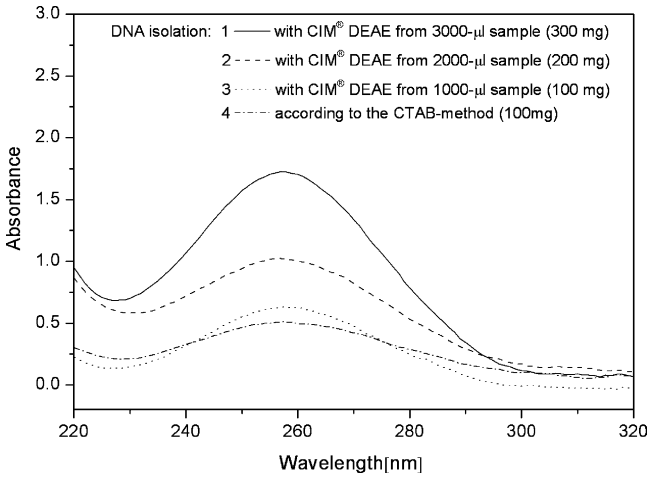

Fig. 7. Absorbance spectra of DNA isolated according to the CTAB-method and with CIM DEAE disks from different quantities of thermally pre-treated corn meal (DNA samples 10× diluted with double distilled water; A_{260}/A_{280}: (line 1) 2.1, (line 2) 1.9, (line 3) 2.5, (line 4) 1.7).

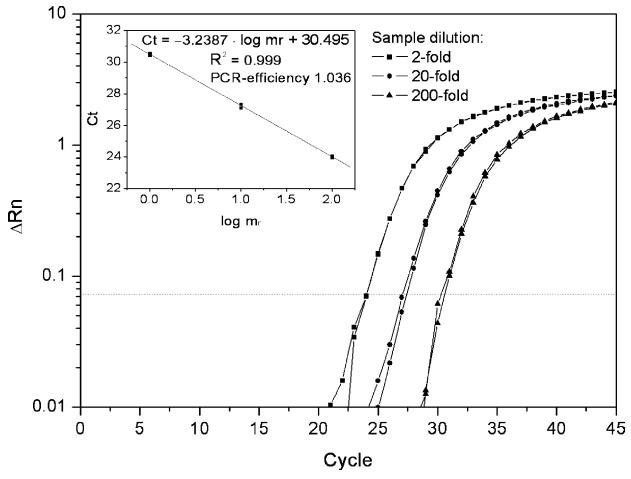

Fig. 8. Real-time PCR amplification plots of DNA isolated with CIM DEAE disks from 300 mg of thermally pre-treated corn meal (primers specific for the maize invertase gene; C_t: cycle threshold; ΔRn: relative fluorescence; m_r: relative quantity).

in contrast to those isolated with the CTAB method (Fig. 6, lane 7). In the smaller samples, only the weak bends of RNA and degraded DNA were visible (Fig. 6, lanes 1–4). After the chromatographic isolation, RNA and DNA remained unseparated. The measurements of the UV spectra demonstrated that protein impurities were successfully removed, because all the ratios between absorbance at 260 and 280 nm were higher than 1.9 (Fig. 7). The concentration of nucleic acids from 100 mg samples was higher in the case of isolation with CIM DEAE disks than with the CTAB method. In comparison with the conventional CTAB method, the steps after the initial extraction consisting of purification with organic solvents and a 60 min precipitation were substituted with a single-step chromatographic isolation. Consequently, the method is less time-consuming and more effective in sense of DNA recovery from the sample.

To determine the purity and the quantity of the DNA, the real-time PCR reactions were performed. DNA was assayed for the invertase gene at three 10-fold dilutions. In Fig. 8, the amplification plots of 2-, 20- and 200-fold diluted sample of DNA isolated with the CIM DEAE disks from 300 mg sample of thermally pre-treated corn meal are presented. The linear regression line of the C_t values against the logarithm of relative DNA quantity had the correlation coefficient of 0.999 indicating linearity of the measurement. The efficiency of the real-time PCR was evaluated from the slope of the linear regression line. The slope of −3.2387 in our case indicated high PCR efficiency, since the slope of −3.322 indicates the maximum PCR efficiency of 1. Both the efficiency and the linearity indicated that the DNA was highly purified, without inhibitors of the PCR. Also the other DNA samples isolated with the CIM DEAE disks from thermally pre-treated corn meal were successfully amplified (data not shown).

The amounts of DNA (Table 1) were estimated with the real-time PCR, based on a calibration curve, where known

Table 1
Quantities of DNA isolated from thermally pre-treated corn meal (DNA quantities were determined with real-time PCR; primers specific for the maize invertase gene; $C_t = -3.3453 \log m + 29.39$, $R^2 = 0.996$)

	DNA isolation method			
	CTAB	CIM DEAE		
Food sample quantity (mg)	100	100	200	300
DNA concentration (ng/µl)	2.0	6.4	8.9	19.8
DNA quantity per sample (ng)	200	640	890	1980

quantities of maize DNA from standard reference material were amplified (data not shown). The quantity of DNA isolated according to the CTAB method from 100 mg sample of thermally pre-treated corn meal was 200 ng, in spite of no visible signal on the electrophoresis (Fig. 6, lane 7). With the CIM DEAE disks, we were able to isolate more than 3-fold the DNA amount from the same quantity of food sample. From 300 mg of the food sample, we isolated up to 1980 ng of DNA. The quantity was therefore adequate for subsequent PCR analyses.

The developed method using CIM disks was tested for the determination of GMO content in a maize sample with known content of GM maize line MON810. DNA was isolated with the CIM DEAE disks from 100 mg sample. Real-time PCR reactions were performed with primers specific for the maize invertase gene and for the GM maize line MON810. The estimated value of GMO content was 0.22% and it was in accordance to the declared value (less than 0.5%).

4. Conclusions

The aim of this study was to investigate the applicability of CIM disk monolithic chromatographic columns for the

isolation of DNA from raw foodstuff and also from processed foods with decreased DNA content for the purposes of GM food detection. It was demonstrated that CIM DEAE columns allow fast and efficient isolation of DNA from raw samples and also from thermally pre-treated corn meal with decreased DNA content. The isolated DNA was of adequate purity and in sufficient quantity for the real-time PCR analyses. The proposed method was also successfully applied for the detection of the GM maize in a food sample. The study expanded the area of possible applications and further developments of the monolithic columns in the area of specific detection methods.

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