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Identification of PCR-Amplified Genetically Modified Organisms (GMOs) DNA by Peptide Nucleic Acid (PNA) Probes in Anion-Exchange Chromatographic Analysis

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PCR products obtained by selective amplification of transgenic DNA derived from food samples containing Roundup Ready soybean or Bt-176 maize have been analyzed by anion-exchange HPLC. Peptide nucleic acids (PNAs), oligonucleotide analogues known to bind to complementary single-stranded DNA with high affinity and specificity, have been used as specific probes in order to assess the identity of the peaks observed. Two different protocols were adopted in order to obtain single-stranded DNA: amplification with an excess of one primer or digestion of one DNA strand. The single-stranded DNA was mixed with the PNA probe, and the presence of a specific sequence was revealed through detection of the corresponding PNA:DNA peak with significantly different retention time. Advantages and limits of this approach are discussed. The method was tested with reference materials and subsequently applied to commercial samples.

KEYWORDS: PNA; PCR products; ion exchange; GMO; DNA; soy; maize

INTRODUCTION

Use of genetically modified organisms (GMOs) in agriculture has become a great subject of debate in recent years (*1*), and as a result, several regulations have been introduced in different countries. In the European Union, labeling of food containing more than 0.9% of GMOs is required and regulations about food and feed labeling and traceability have been approved (*2*). Therefore, the need for fast, simple, and sensitive methods for detection and quantification of GMOs is increasingly important in food control (*3*). Although analysis of proteins can be used for these purposes, detection of specific DNA sequences by polymerase chain reaction (PCR) is the most widely used technique (*4*) due to the higher stability and traceability of DNA in both raw material and processed food matrices.

PCR methods can sometimes generate ambiguous interpretations of the results due to either "carry-over" contaminations or low specificity of the primers leading to false positives. For example, the presence of transgenic maize in Mexico has been debated in terms of the possibility of false priming (*5*). Post-PCR control has been shown to be often essential to confirm a sequence identity in the case of ambiguous recognition of specific targets (*6*). Nested PCR methods (*7*), real-time quantitative PCR (*8*), biosensor technology (*9*), and microarray technology (*10, 11*) were developed for increasing the specificity of GMO analysis. All these techniques allow obtaining more reliable results provided that the risk of contamination or "carry over" is carefully avoided.

Use of highly specific probes for confirmation of the identity of PCR products is particularly important where the presence of GMO has immediate industrial or legal consequences (for example, in summer 2003, hundreds of hectares of maize contaminated with small percentages of GM varieties were destroyed or withdrawn in northern Italy (*12*)).

One of the most specific classes of probes used for DNA detection is that of peptide nucleic acids (PNAs, **Figure 1**), oligonucleotide mimics in which the sugar−phosphate backbone has been replaced by a pseudopeptide chain of *N*-(2-aminoethyl)glycine monomers (**Figure 1**). PNAs were shown to bind to complementary DNA or RNA sequences with higher affinity and specificity than conventional DNA probes (*13*−*15*).

On account of these properties, PNAs have been used for detection of specific gene sequences in advanced diagnostic methods (*16*−*25*). Single-molecule detection of transgenic DNA was also performed by means of PNA probes and double-wavelength fluorescence analysis (*26*).

In a previous work, we have shown how hybridization of oligonucleotides with PNA can be detected using anion-exchange HPLC, which operates under nondenaturing conditions (*27*). In the present work, we report the development of a protocol for assessment of the identity of PCR products derived from genetically modified soybean and maize using PNA hybridization and anion-exchange HPLC. PNAs of different length were used for gene sequences corresponding to a region of the CaMV 35S-CTP construct of Roundup Ready (RR) GM-

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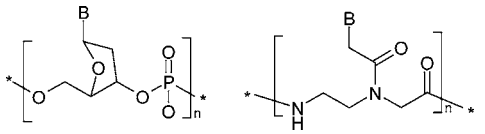

Figure 1. Structure of DNA (left) and PNA (right). B = nucleobase.

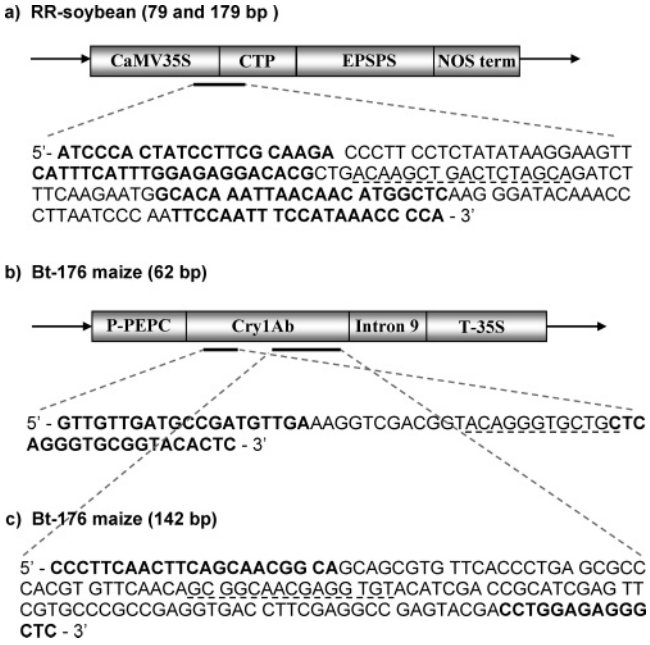

Figure 2. Sequences of the DNA tracts amplified by PCR for (**a**) the Roundup Ready soybean construct and the 79 bp amplicon used in the present study, (**b**) Bt-176 maize construct and the 62 bp amplicon used in the present study, and (**c**) 142 bp amplicon of the same Bt-176 construct. Bold characters represent the position of primers, and underscored bases represent the position of the target for the PNA probes. RR soybean external primers give a 169 bp amplicon; internal primers give a 79 bp amplicon. Two regions of the Cry1Ab sequence (62 and 142 bp) were amplified for detection of Bt 176 maize. (CaMV35S, cauliflower mosaic virus 35S promoter; CTP, chloroplast transit peptide; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; NOS, nopaline synthase; P-PEPC, phosphoenolpyruvate carboxylase promoter; Cry1Ab, Cry1Ab delta-endotoxin (*Btk* HD-1); T-35S, CaMV 35S terminator.)

soybean and the CryIA tract of the Bt-176 GM-maize, whose locations within the transgenic construct are reported in **Figure 2**, in order to evaluate the effect of PNA structure on signal intensity and specificity.

EXPERIMENTAL PROCEDURES

General. PNA monomers were from Applera (Milan, Italy); (4-methylbenzhydryl)amine (MBHA) resin was from Novabiochem (Inalco spa, Milan, Italy); *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and *N*,*N*-diisopropylethylamine (DIEA) were from Aldrich (Milan, Italy), and *N*-methylpyrrolidone (NMP) was from Advance Biotech Italia srl (Seveso, Italy).

All solvents used for HPLC were of chromatographic grade. Doubly distilled water was produced by the Millipore Alpha-Q purification module.

Oligonucleotides used for melting temperature measurement were purchased from Genset (Paris, France) and Thermoelectron (Ulm, Germany), and their purity was checked by ion-exchange HPLC.

Standard soybean and maize flour of known GMO content were obtained from Fluka (Milan, Italy). The commercial samples used were the same as those previously described (*28*), and their GMO content (w/w %) was measured by real-time PCR (*29*).

DNA Extraction. DNA extraction was performed using the Wizard Plus Minipreps System (Promega Italia, Milan, Italy) following the manufacturer's specifications with modification: lipids were removed by extraction with chloroform in an additional purification step. The extraction procedure is summarized below: 0.2 g of ground sample was transferred to a 2 mL Eppendorf tube and mixed with 500 μL of extraction buffer (10 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% SDS, pH 8) and 500 μL of 5 M guanidine·HCl and incubated at 60 °C for 3 h. Lipids were removed from each sample by extraction with 500 μL of chloroform followed by centrifugation at 16000*g* for 10 min. The supernatant was then collected in 2 mL plastic tubes, and 8 μL of Proteinase K (20 mg/mL) was added to each tube; the samples were incubated at 60 °C for 10 min and then centrifuged at 16000*g* for 1 min. The supernatant was transferred to a new 2 mL plastic tube, and 5 μL of RNase (100 mg/mL) was added; digestion was carried out at 40 °C for 10 min, 500 μL of this solution was transferred to a 2 mL Eppendorf tube, and 1 mL of DNA-binding resin (Wizard Plus Minipreps DNA purification system, Promega Italia, Milan, Italy) was added and thoroughly mixed by inverting several times. The resin was then transferred to a minicolumn using a 2 mL syringe and subsequently washed with 2 mL of 80% isopropyl alcohol according to the manufacturer's instructions. The purified DNA was eluted from the column with 100 μL of prewarmed water (70 °C) and stored at −20 °C before use. The DNA concentration was measured by UV absorption at 260 nm, while the DNA purity was evaluated based on the UV absorption ratio at 260/280 nm. All samples showed a 260/280 nm ratio ranging from 1.6 to 2.0. The extracted materials were then diluted to a final concentration of 50 ng/μL and used as stock solutions for PCR analysis.

PCR Methods. All primers used were purchased from Sigma Genosys (Cambridge, U.K.). Amplification for development of the PCR method was performed using standard primers, while that used for HPLC assessment was performed with opportunely functionalized primers: the target strand was copied by a primer labeled with a cyanine (Cy5 or Cy3) fluorophore at the 5′ end, while the nontarget strand was copied using a 5′ phosphorylated primer.

Polymerase chain reactions were performed in a final volume of 50 μL. All reagents were supplied by Euroclone (Milan, Italy). The concentrations of the reagents in the final mix were as follows: 1× reaction buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of each primer, 0.05 u/μL of hot start Blue Taq, and 300 ng of DNA. The PCR was carried out in a PCR-sprint Thermal Cycler (Thermo Hybaid, Basingstoke, U.K.) using the following conditions: 1 cycle of DNA denaturation and Blue Taq activation at 95 °C for 5 min; 40 cycles consisting of DNA denaturation at 95 °C for 50 s, primers annealing at 60 °C for 50 s, and elongation at 72 °C for 50 s; one step of final elongation at 72 °C for 5 min. The PCR products were immediately analyzed by 2% agarose gel in 0.5× TBE or stored at −20 °C until use. In the double-unbalanced PCR a first amplification was performed as described above. A small amount (2−5 μL) of the reaction mixture was then amplified in the second run using a fresh Mastermix solution of the same composition except that a 10-fold excess of the fluorescent primer (or only the fluorescent primer) was used.

PNA Synthesis. The PNA was synthesized using solid-phase synthesis with a 433A Peptide Synthesizer (Applied Biosystems) with the BOC strategy and HATU/DIEA coupling, as described in a previous work (*18*). Swelling, downloading, and cleavage of the PNAs from the resin were done manually.

The crude product was purified by reversed-phase HPLC using a Phenomenex C18 peptide column (3 μm, 250 mm × 10 mm) with a binary gradient (flow rate, 4 mL/min). Eluent A: water/TFA = 100:0.2. Eluent B: water/acetonitrile/TFA = 60:40:0.2. Detector: UV (260 nm). The purified product was then identified by mass spectrometry (Micromass ZMD). **PNA 1** calcd *m*/*z* 1118.2 (MH_4^{4+}), 894.7 (MH_5^{5+}), 745.8 (MH_6^{6+}), 639.4 (MH_7^{7+}); found 1118.4, 894.8, 745.8, 639.3. **PNA 2** calcd *m*/*z* 1364.7 (MH_3^{3+}), 1023.8 (MH_4^{4+}), 819.2 (MH_5^{5+}), 682.8 (MH_6^{6+}); found 1364.4, 1023.7, 819.0, 682.7. **PNA 3** calcd *m*/*z* 1461.3 (MH_3^{3+}), 1096.2 (MH_4^{4+}), 877.2 (MH_5^{5+}), 731.1 (MH_6^{6+}), 626.8 (MH_7^{7+}); found 1461.4, 1096.5, 877.2, 731.3, 627.0. **PNA 4** calcd *m*/*z* 1365.3 (MH_3^{3+}), 1024.2 (MH_4^{4+}), 819.6 (MH_5^{5+}), 683.2 (MH_6^{6+}); found 1365.3, 1024.0, 819.6, 683.3. **PNA 5** calcd *m*/*z* 1077.1 (MH_3^{3+}), 808.1 (MH_4^{4+}), 646.7 (MH_5^{5+}), 539.0 (MH_6^{6+}); found 1077.1, 808.1, 646.6, 539.0. **PNA 6** calcd *m*/*z* 1333.7 (MH_3^{3+}), 1000.5 (MH_4^{4+}), 800.6

Table 1. Primers Used in the Present Study

Name	Target DNA	Sequence	Amplicon name / length
RR474[a]	Roundup Ready	5'-CATTTCATTTGGAGAGGACACG-3'	DNA **1** / 79bp
RR396[b]		5'-GAGCCATGTTGTTAATTTGTGC-3'	
GM07[a]	Roundup Ready	5'-ATCCCACTATCCTTCGCAAGA-3'	DNA **2** / 169bp
GM08[b]		5'-TGGGGTTTATGGAAATTGGAA-3'	
CR145[a]	Bt-176	5'-GTTGTTGATGCCGATGTTGA-3'	DNA **3** / 62bp
CR84[b]		5'-GAGTGTACCGCACCCTGAG-3'	
BT176F[a]	Bt-176	5'-CCC TTC AAC TTC AGC AAC GGCA-3'	DNA **4** / 142bp
BT176R[b]		5'-GAG CCC TCT CCA GGT GGT A-3'	

[a] Cy3 or Cy5 labeled at 5′ end [b] Phosphorylated at 5′ when digestion was performed.

Table 2. PNA Sequences Used in the Present Study

Name	Target DNA	Sequence	Tm[b] (°C)
PNA 1	Roundup Ready	DNS- AEEA[a]-TGCTAGAGTCAGCTT-NH_2	77
PNA 2	Roundup Ready	H-TGCTAGAGTCAGCTT-NH_2	75
PNA 3	Roundup Ready	H-$(AEEA)_2$[a]-TGCTAGAGTCAGCTT-NH_2	79
PNA 4	Roundup Ready	H-CTA GAG TCA GCT TGT-NH_2	73
PNA 5	Bt 176 maize	H-GCA GCA CCC TGT-NH_2	69
PNA 6	Bt 176 maize	H-$(AEEA)_2$[a]- ACACCTCGTTGCCGC-NH_2	76
PNA 7	scrambled	H-$(AEEA)_2$[a]-ATGATTTCAATGCTC.NH_2	61

[a] AEEA = 2-[2-(2-aminoethyl)ethoxy]acetyl spacer. [b] Melting temperatures of the PNA:DNA duplex measured at c = 5 μM for each strand.

(MH_5^{5+}), 667.3 (MH_6^{6+}); found 1333.9, 1000.8, 800.8, 667.4. **PNA 7** calcd m/z 1085.9 (MH_4^{4+}), 869.0 (MH_5^{5+}), 724.3 (MH_6^{6+}), 621.0 (MH_7^{7+}); found 1086.5, 869.6, 724.9, 621.5.

Melting Temperature Measurements. Stock solutions of PNA and DNA were prepared in doubly distilled water, and their concentration was measured by absorbance using the following ϵ_{260} (M^{-1} cm^{-1}) for the nucleobases: thymine (T) 8800, cytosine (C) 7300, adenine (A) 10 400, guanine (G) 11 700.

All hybrid samples reported (5 μM for each strand) were first incubated at 90 °C for 5 min and then slowly cooled at room temperature. All hybridization experiments were carried out in a 10 mM phosphate buffer, 100 mM NaCl, 0.1 mM EDTA, and pH 7. Melting curves were recorded on a Perkin-Elmer λ BIO 20 spectrophotometer equipped with a PTP 6 Peltier temperature programmer and a cell changer. The samples were heated (1 °C/min, 5 points/min), and the UV signal variation at 260 nm was recorded. Melting temperatures were taken as the maxima of the first derivative of the melting curves.

HPLC Analysis. PCR products labeled with both the fluorescent Cy5 (or Cy3) and the phosphate group were used for the HPLC assay using anion-exchange HPLC with fluorescent detection. PCR products were first purified using the GenElute PCR Clean Up Kit (Sigma-Aldrich, Milan, Italy) according to the manufacturer's instructions. The purified double-stranded product then underwent enzymatic digestion by Lambda Exonuclease (USB Corp., Amersham Biosciences Europe, Milan, Italy) which recognizes and selectively digests 5′ phosphorylated DNA strand in a dsDNA; reaction mix concentrations were as follows: 1× Lambda Exonuclease reaction buffer, 10 units of enzyme, and 500 ng of dsPCR product in a final volume of 25 μL. The digestion was carried out for 45 min at 37 °C.

The single-stranded PCR product obtained by digestion, containing only the target strand, was then hybridized with the complementary PNA probe in a mixture containing 20 μL of ssDNA and 200 pmol of PNA (large excess) in a final volume of 30 μL. The mixture was heated at 95 °C for 5 min and then cooled at 25 °C. The products obtained were analyzed by anion-exchange HPLC. All experiments were carried out using an Alliance 2690 Separation Module HPLC system (Waters), equipped with a temperature controller, Dual λ Absorbance Detector 2487 (Waters), and Scanning Fluorescent Detector 474 (Waters). Anion-exchange chromatographic measurements were carried out using the column TSK-gel DNA-NPR 4.6 mm i.d. × 7.5 cm (column 1) and TSK-gel DEAE-NPR 4.6 mm i.d. × 3.5 cm (column 2) (Tosoh Biosep) with UV (λ = 260 nm) and fluorescence detection (λ = 646 and 664 nm). Binary linear gradients were as follows: eluent A = Tris 0.02M pH 9; B = Tris 0.02 M, NaCl 1 M, pH 9; flow = 0.5 mL/min; T = 35 °C. Gradient 1: elution from 50% A and 50% B to 30% A and 70% B in 10 min. Gradient 2: elution from 60% A and 40% B to 30% A and 70% B in 15 min. Gradient 3: from 55% A to 50% A in 4 min then to 36% A in 21 min.

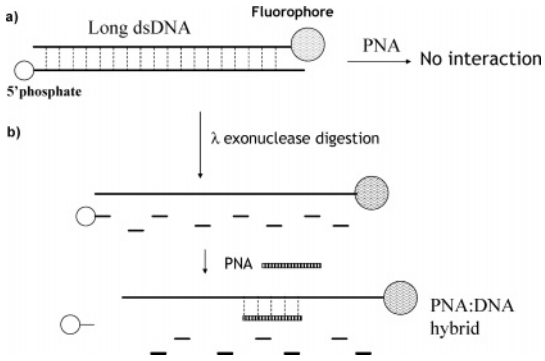

Figure 3. Schematic representation of the treatment used for identification of PCR products.

Analyses of dsDNA under denaturing conditions were performed using the above-described eluents containing either 2 M urea or 10% formamide.

For evaluation of the detection limit, the synthetic 79 DNA **1** oligomer characteristic of Roundup Ready construct fluorescently labeled at 5′ with Cy5 was used. Samples of 10 different concentrations (in triplicate) ranging from 0 to 500 nmol/L were injected (10 μL injection).

For evaluation of the detection limits in terms of % GMO, certified standard sets of Roundup Ready soy and Maize Bt176 were extracted, amplified with 40 cycles, purified, and digested as reported above (in duplicate). All samples showed the expected HPLC peak (using column

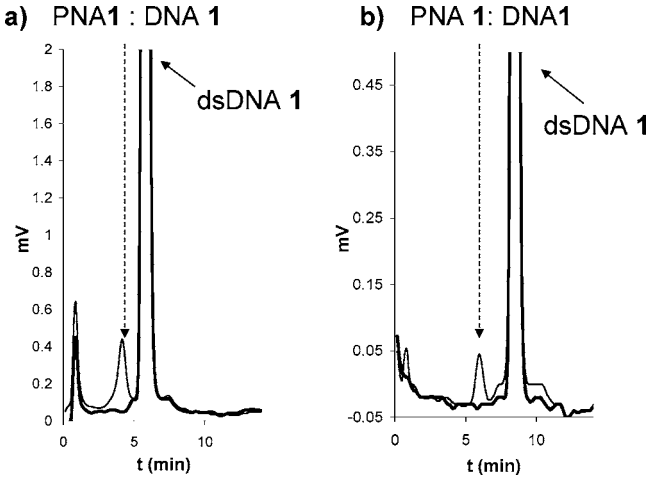

Figure 4. IE-HPLC chromatograms obtained with dsPCR amplicon (DNA **1**, 79bp) from Roundup Ready soybean using (**a**) 2 M urea and (**b**) 10% formamide as additive to the eluent. Column: TSK gel DEAE-NPR (4.6 mm i.d. × 3.5 cm). T = 35 °C. Eluents: (A) Tris 20 mM in H_2O at pH 9 containing either urea of formamide; (B) NaCl 1 M in eluent A at pH 9. Gradient: from 50% A to 30% A in 10 min. Flow = 0.5 mL/min. Fluorescence detector: λ_{ex} = 646 nm and λ_{em} = 664 nm. PNA **1** was used as probe.

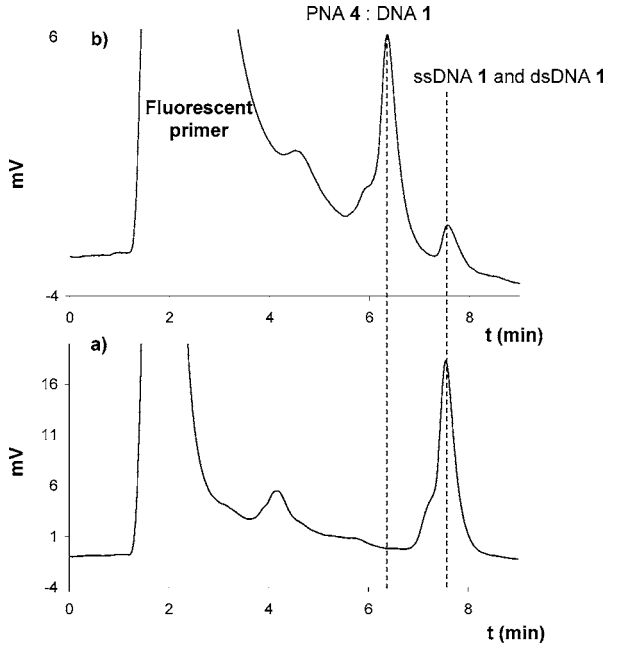

Figure 5. HPLC analysis of PCR mixtures obtained by unbalanced PCR (79 bp) in the absence (**a**) and presence (**b**) of PNA **4** probe. Column: TSK gel DEAE-NPR (4.6 mm i.d. × 7.5 cm). T = 25 °C. Eluents: (A) Tris 20 mM in H_2O at pH 9; (B) NaCl 1 M in eluent A at pH 9. Gradient: from 50% A to 30% A in 10 min. Flow = 0.5 mL/min. Fluorescence detector: λ_{ex} = 646 nm and λ_{em} = 664 nm.

1 and gradient 1 reported above), while the blank (0% GMO) did not give a distinguishable signal.

RESULTS AND DISCUSSION

1. PCR Amplicons and PNA Probes. The PNA sequences were chosen in an internal part of the PCR amplicons used for identification of transgenic Roundup Ready soybean construct for the CryIA gene (contained in Bt-176, Bt-11, and MON810 transgenic maize) and Bt 176 maize constructs. The sequences of the amplicons used, location of the primers, and position of

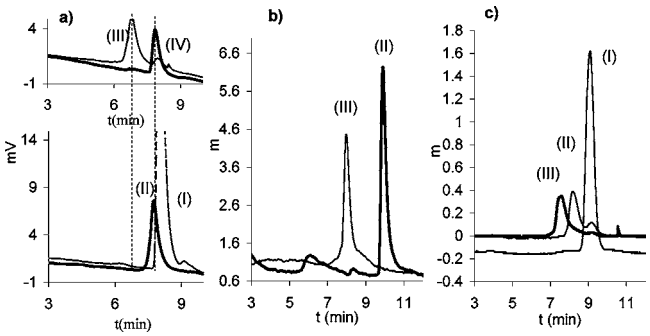

Figure 6. IE-HPLC analysis of Roundup Ready amplicons. (**a**) 79 bp Cy-5-labeled PCR product: (I) dsDNA **1** purified; (II) ssDNA **1** from digestion; (III) PNA **4**:DNA hybrid; (IV) digested ssDNA **1** mixed with noncomplementary PNA **7**. (**b**) 169 bp Cy-5-labeled PCR product: (II) ssDNA **2** from digestion; (III) PNA **4**:DNA **2** hybrid. Column: TSK gel DNA-NPR (4.6 mm i.d. × 7.5 cm). T = 35 °C. Eluents: (A) Tris 20 mM in H_2O at pH 9; (B) NaCl 1 M in eluent A at pH 9. Gradient: from 100% A to 0% A in 20 min. Flow = 0.5 mL/min. Fluorescence detector: λ_{ex} = 646 nm and λ_{em} = 664 nm. (**c**) 62 bp (DNA **3**) Cy-5-labeled PCR products from Bt-176: purified dsDNA **3** (I), ssDNA **3** from digestion (II), and PNA **5**:DNA **3** hybrid (III). Column TSK gel DEAE-NPR (4.6 mm i.d. × 3.5 cm). T = 35 °C. Eluents: (A) Tris 20 mM in H_2O at pH 9, (B) NaCl 1 M in eluent A at pH 9. Gradient: from 60% A to 30% A in 15 min. Flow = 0.5 mL/min. Fluorescence detector: λ_{ex} = 646 nm and λ_{em} = 664 nm.

the PNA target sequence are reported in **Figure 2**. The primers used together with their sequence, target, and amplicon length are reported in **Table 1**, and PNA sequences are listed in **Table 2**. The primers Bt-176 F and Bt-176 R are located in an internal part of the CryIA gene of Bt-176 maize and were found to be selective for Bt-176 maize (results not shown).

The PNA probes were synthesized using solid-phase methodologies on an automatic peptide synthesizer, purified by preparative RP-HPLC, and identified by their ESI mass spectra. Using a 5 μmol scale, the product obtained was enough for a large number (>1000) of tests.

The PNA probes were hybridized with complementary oligonucleotides of the same length in order to evaluate the PNA:DNA duplex stabilities (melting temperatures, T_m) (**Table 2**). The melting temperatures were found to be dependent on the length and base composition of the PNA probes, in agreement with literature data (*30*), but were only slightly affected by the presence of groups in the amino terminal part (either a fluorophore or a simple spacer). For the Roundup Ready target DNA, the melting temperature was also evaluated using a single-stranded synthetic oligonucleotide corresponding to the 79 bp amplicon described in **Figure 2**; the T_m observed in this case (80 °C) was comparable with that observed with the short oligonucleotide.

2. HPLC Analysis and Protocols. *Hybridization Experiments with Long Oligonucleotides.* In a previous work (*27*), we demonstrated that by using anion-exchange chromatography it was possible to modify the retention times of synthetic short oligonucleotides (11-mers) using peptide nucleic acids (PNAs) of the same length and with complementary sequences. The effect was attributed to formation of stable PNA:DNA duplexes, which have a different retention behavior than DNA alone, due to the different size/charge ratio. It was therefore interesting to verify if the same type of analysis could be performed using long oligonucleotides as targets, which could mimic the interaction with long PCR products. For this purpose we utilized a nonporous hydrophilic anion-exchange column (TSK gel), which is particularly suitable for DNA analysis, allowing minimizing

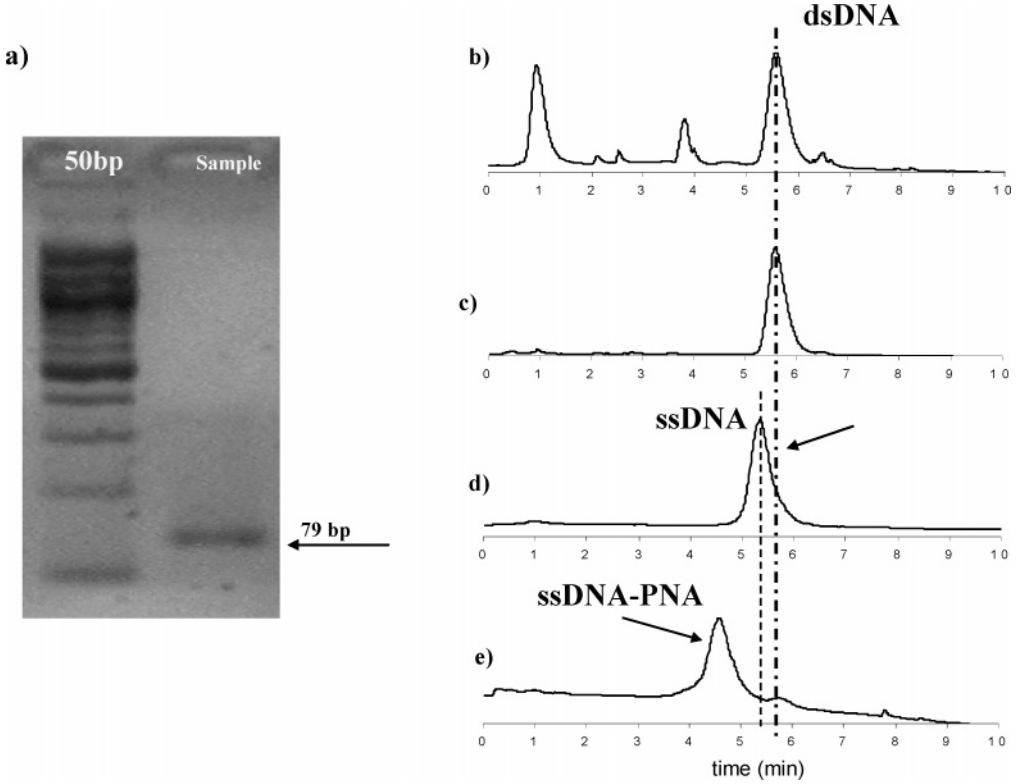

Figure 7. Agarose gel-electrophoresis analysis (**a**) and HPLC profiles (**b**–**e**) of PCR amplicon specific for RR soybean (79 bp) obtained from a soy burger labeled "GMO-free". (a) 50 bp refers to a 50 bp DNA ladder. Sample: PCR product of the 79 bp amplicon for RR soy. (Right) HPLC profile of the PCR product crude (**b**), after purification (**c**), after digestion with λ-exonuclease (**d**), and after hybridization with PNA **3** (**e**). Column: TSK gel DEAE-NPR (4.6 mm i.d. × 3.5 cm). T = 35 °C. Eluents: (A) Tris 20 mM in H_2O at pH 9; (B) NaCl 1 M in eluent A at pH 9. Gradient: from 50% A to 30% A in 10 min. flow = 0.5 mL/min. Fluorescence detector: λ_{ex} = 646 nm and λ_{em} = 664 nm.

the hydrophobic interactions of the nucleobases with the stationary phase (*31*).

Hybridization experiments using a Cy5-labeled synthetic oligonucleotide of the same length and sequence of the PCR amplicon designed for Roundup Ready soybean (DNA **1**) were performed. A PNA 15-mer complementary to an internal sequence of this DNA labeled with a dansyl group was used (PNA **1**). Also, in this case, a new peak with a retention time (t = 3.9 min using gradient 1) different from that of DNA alone was observed (t = 4.9 min, under the same conditions), which was attributed to the PNA:DNA duplex. Identification was confirmed by observation of the fluorescence emission of both the Cy5 fluorophore on the oligonucleotide (λ_{ex} = 646 nm and λ_{em} = 664 nm) and the dansyl group on the PNA probe (λ_{ex} = 330 nm and λ_{em} = 530 nm) in the same peak. Since these two fluorophores have excitation and emission wavelengths completely different, this observation demonstrated that the new peak formed contains both DNA and PNA and therefore corresponds to the PNA:DNA hybrid. This interpretation was confirmed by experiments carried out at higher temperatures: a progressive decrease of the PNA:DNA peak and a corresponding increase of the DNA peak were observed (results not shown).

Use of a noncomplementary PNA (i.e., with a random sequence) did not produce formation of a new peak. We thus concluded that the appearance of the new PNA:DNA peak was sequence specific and could be used for detection of the specific DNA sequence complementary to the PNA used.

Hybridization Experiments with PCR Products. Hybridization of the PNA **1** or PNA **2** probes with double-stranded (ds) PCR product obtained by amplifying a Roundup Ready soybean tract did not produce any new peak. Therefore, the short PNA probe was not able to displace a long DNA strand from its target (**Figure 3a**).

Slightly better results were obtained using denaturing agents in the eluent, such as formamide or urea, but only a small fraction (6%) of the dsDNA gave rise to the PNA:DNA duplex (**Figure 4**), which was identified by comparison with that obtained with single-stranded DNA under the same conditions.

In another approach, unbalanced PCR, i.e., amplification of DNA using an excess of the fluorescently labeled primer, was used in order to obtain an excess of single-stranded labeled target DNA. The resulting single-stranded DNA was successfully hybridized with PNA **1** (**Figure 5**), giving rise to the peak corresponding to the PNA:DNA complex, which was identified by its retention time. Though this approach is relatively simple and does not require further treatment of the PCR samples, the presence of many components in this type of samples with strong peaks of dsDNA and the fluorescent primer in excess makes this approach less suitable for HPLC analysis.

We therefore decided to produce single-stranded PCR products by digesting the strand complementary to the DNA target. For this purpose, the PCR reaction was performed using a 5′-Cy5-labeled primer on the target strand and a 5′-phosphorylated primer for the complementary strand (**Figure 3b**). After amplification, the DNA obtained was purified, using a GenElute PCR Clean Up Kit, and then digested with λ-exonuclease, which cleaves only the strand containing the phosphorylated primer. The single-stranded DNA thus obtained is identical to the synthetic oligonucleotide used in the previous section and therefore easily hybridized with the PNA probe. Using this protocol, the DNA extracted from Roundup Ready soy flour was amplified and digested, giving a 79 nucleotide (nt) single-

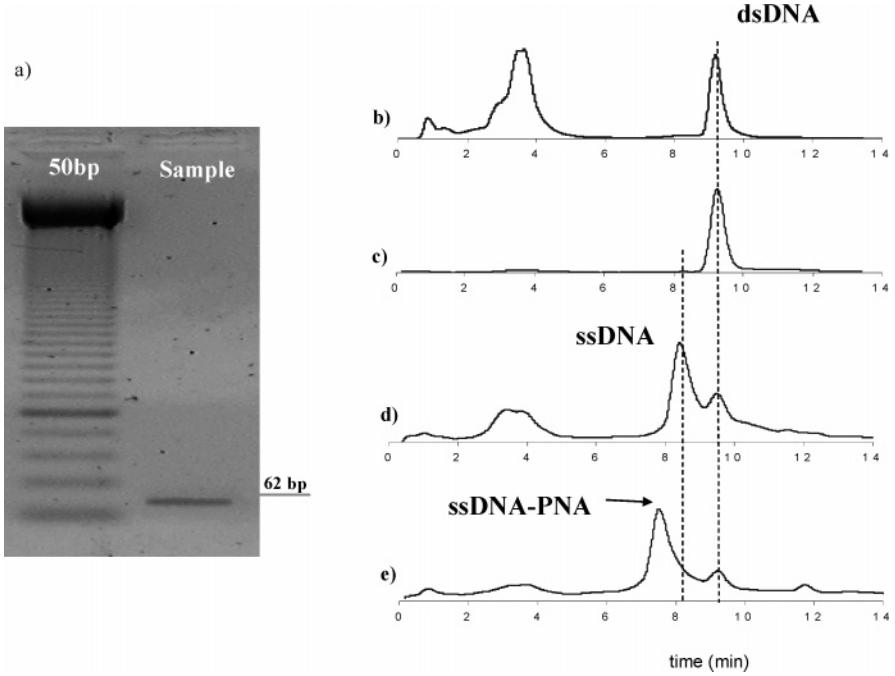

Figure 8. Agarose gel-electrophoresis analysis (**a**) and HPLC profiles (**b**–**e**) of PCR amplicon specific for Bt-176 maize (62bp) obtained from "organic" biscuits. (**a**) 50 bp refers to a 50 bp DNA ladder. Sample: PCR product of the 62 bp amplicon for RR soy. (Right) HPLC profile of the PCR product crude (**b**), after purification (**c**), after digestion with λ-exonuclease (**d**), and after hybridization with PNA **5** (**e**). Column: TSK gel DEAE-NPR (4.6 mm i.d. $\times$ 3.5 cm). $T = 35$ °C. Eluents: (A) Tris 20 mM in H_2O at pH 9; (B) NaCl 1M in eluent A at pH 9. Gradient: from 50% A to 30% A in 10 min. Flow = 0.5 mL/min. Fluorescence detector: $\lambda_{ex} = 646$ nm and $\lambda_{em} = 664$ nm.

stranded DNA, which was analyzed by ion-exchange HPLC (**Figure 6a**, lower part), and assessment of the identity of the observed peak was performed using either PNA **2** or PNA **4** as probe (**Figure 6a**, upper part).

Use of a noncomplementary probe (PNA **7**, **Figure 6a**, upper part) did not produce changes in the DNA retention time, confirming the specificity of the assay. The same approach was also used for assessment of the identity of a longer amplicon (169 nt) derived from Roundup Ready soybean with PNA **4** (**Figure 6b**). Use of longer amplicons has the advantage of increasing the performance in the purification step; the difference in the retention time between PNA and PNA:DNA duplex is larger using this DNA size, though the analysis time is slightly longer ($t = 9.9$ min for ssDNA and $t = 7.9$ min for PNA:DNA). Use of a shorter column for the separation allows reducing the analysis time ($t = 7.7$ min for ssDNA and $t = 8.2$ min for PNA:DNA) but with lower resolution. Similar results were obtained with PNA **2**. Using Cy3-labeled ssDNA **2** (169 bp) and PNA **1** it was possible also in this case to show that the anticipated peak contains both Cy3 and the dansyl fluorescence.

Analysis of ssDNA amplified from Bt-176 maize (62 nt and 142 nt) was performed using PNA **5** and **6**, respectively, although in this case the gradient used was modified in order to better separate the peaks (gradient 2 in the Experimental Procedures). In the chromatogram reported in **Figure 6c**, the identification of DNA **3** using the same approach is reported. The double-stranded DNA (I) amplicon (62 bp) and the digested ssDNA (II) were separated, allowing evaluating the efficiency in the digestion step (some undigested dsDNA is still visible in this example). Hybridization of ssDNA with PNA **5** gave a shorter retention time, corresponding to formation of the PNA **5**:DNA **3** hybrid.

The same result was obtained with the longer DNA **4** (142 nt) hybridized with PNA **6** with slightly different conditions (column 1 and gradient 3, see Experimental Procedures); the retention times of dsDNA and ssDNA were very close (6.9 min for ssDNA and 7.1 min for dsDNA), but the PNA **6**:DNA **4** peak was separated ($t = 6.4$ min).

In general, recovery of DNA after digestion was higher for the longer DNA **2** and **4**, giving rise to more intense peaks in the chromatographic analysis.

Evaluation of Method Performances. The limit of detection was evaluated in terms of both the minimum quantity of DNA which can be detected and the minimum GMO percentage.

For evaluation of the DNA detection limit, a Cy5-labeled synthetic oligonucleotide 79mer identical to DNA **1** was used, and a linear calibration curve was obtained with $R^2 = 0.9974$ (10 samples in triplicate). The limit of detection (LOD) was found to be 0.2 pmol, corresponding to a 20 nM concentration (volume of injection = 10 μL). Therefore, in absolute terms, the HPLC method is very sensitive and comparable to other advanced methodologies.

However, since the HPLC method is used in combination with PCR, preparation of the samples has a large influence on the performance and both variability and low recovery are possible. We therefore evaluated the detection limit in terms of % GMO using standard certified reference material treated with the same protocols reported above (extraction, amplification, purification, and digestion). Since all positive samples gave significant (S/N > 10) chromatographic peaks, the detection limit of the entire method is $\leq$0.1% for both RR soy (79 and 169 nt) and Bt176 maize (62 and 142 nt).

In these samples, using the same number of PCR cycles, the 0.1% sample did not give rise to a clear band in the gel electrophoresis analysis in the case of RR-soy amplicon of 169 bp, while for the RR 79 bp and the maize Bt 62 bp and Bt 142 bp amplicon the detection limit was similarly $\leq$0.1%.

False negatives were found in cases where the PCR reaction failed, but the rate of these is obviously the same as in the PCR-gel electrophoresis analysis. In addition, in some of the tests

(9/56, 16%) hybridization with complementary PNA was not observed due to the lack of digestion, indicating that this step has to be carefully carried out. The protocol involving digestion of one strand is dependent on the integrity of the phosphorylated standard and the λ-exonuclease. Careful treatment of the nuclease and checking the integrity of the primer is therefore a requisite for use of this protocol. Identification by HPLC of the single-stranded DNA peak (even if the digestion is not complete) is a prerequisite for subsequent PNA hybridization.

On the contrary, no false positive was detected, since, as reported in **Figure 6**, the hybridization is highly sequence specific, if the samples are correctly treated. However, a few cases (4/60, 7%) of false positive due to carry over were detected, but these were identified by careful replicate analysis. On the contrary, using the PCR-electrophoresis method, some examples of false positive were reported in the literature (*32*) and in our experience, especially in the case of screening made on the 35S promoter sequence. Furthermore, for some of the amplicons reported in this paper, the size of RR soy and maize Bt are comparable (79 bp and 62 bp), and simultaneous analysis of the two bands, such as in multiplex PCR, is strongly dependent on the separation efficiency and relative quantity, while in the HPLC-PNA method, based on a different principle (sequence-specific hybridization instead of size), no crossed attribution was obtained.

It should be stressed that the above considerations are the results of data available from our research during method development, while more precise evaluation of LOD and false positive and false negative rates should be the subject of interlaboratory ring test, which is beyond the scope of the present work.

Application of the Method on Two Commercial Samples. Finally, as an example of the possible applicability of this analysis to food products, we used the method described above to assess the band identity in two commercial food samples in which in a previous study (*28*) a positive band was found using multiplex PCR analysis. The first was DNA extracted from a soy burger containing 40% (w/w) of soy proteins and labeled "GMO free". PCR amplification gave rise to the expected 79 bp amplicon (**Figure 7a**), which was also detected in the HPLC system (**Figure 7b**). After purification of DNA, the HPLC profile was clearly improved (**Figure 7c**). Digestion of DNA gave rise to the ssDNA peak (**Figure 7d**); hybridization with PNA 3 induced formation of the PNA:DNA adduct, which was identified by its retention time (**Figure 7e**). The residual peak observed after hybridization could be attributed to a small fraction of undigested DNA. Independent measurements by real-time PCR confirmed that the sample contained 0.6% (w/w) of RR soybean (*28*).

The second sample was derived from biscuits containing maize flour and maize malt labeled as derived from "organic" production (**Figure 8**), which was found to be positive in PCR amplification specific for the Bt176 maize (**Figure 8a**). Also, in this case, HPLC analysis confirmed that the amplicon contained the specific sequence complementary to the PNA probe (PNA **5**), indicating that the observed band in gel electrophoresis was not due to a false positive. Real-time PCR analysis of this sample revealed that the sample was contaminated with less than 0.25% (w/w) of Bt-176 maize (*28*).

3. Conclusions. PNA can be used in combination with ion-exchange high-performance liquid chromatography for assessment of the identity of PCR amplicons to perform a highly sequence-specific test. Analysis can be performed with instrumentation (HPLC) that is widely used in chemical laboratories using two simple purification steps and a digestion step after the normal PCR protocol and is very fast (5−10 min each injection) if compared with normal gel electrophoresis analysis. The results obtained using the very specific PNA probes are very sound provided that, as in other PCR-based DNA detection methods (including real-time PCR), contamination of the samples is carefully avoided and digestion is effectively carried out.

The method proposed has been used in the present work for assessment of the presence of transgenic materials in food but can also be applied to other diagnostic problems (both in food science and in biomedical research). The results obtained using this approach on GMOs can be important for samples in which the presence of DNA from undesired contaminants, even in trace amounts, has commercial or legal consequences, as for GMOs in several products (e.g., in seeds, infant products, "GMO-free"-labeled products, or organic foods).

The PNA probes used have two advantages: they are superior to oligonucleotides in terms of sequence selectivity and, due to their neutral backbone, can shift the IE-HPLC DNA peak more efficiently, giving rise to a distinct peak.

Use of HPLC also allows one to envisage the possibility of quantification of the DNA from PCR by quantitative analysis of the peak area. Work is in progress to evaluate the possibility of combining quantitative HPLC analysis and assessment of the identity by very specific PNA probes.

One of the most interesting applications of the present method would be detection of single point mutations which are not detected by the PCR-gel electrophoresis method and with hardly any other advanced PCR techniques but are efficiently recognized by the PNAs (*33*) and especially modified PNA with enhanced sequence specificity (*34*−*36*).

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