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Dipstick Test for DNA-Based Food Authentication. Application to Coffee Authenticity Assessment

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ABSTRACT: This paper reports DNA-based food authenticity assays, in which species identification is accomplished by the naked eye without the need of specialized instruments. Strongly colored nanoparticles (gold nanoparticles) are employed as reporters that enable visual detection. Furthermore, detection is performed in a low-cost, disposable, dipstick-type device that incorporates the required reagents in dry form, thereby avoiding multiple pipetting and incubation steps. Due to its simplicity, the method does not require highly qualified personnel. The procedure comprises the following steps: (i) PCR amplification of the DNA segment that flanks the unique SNP (species marker); (ii) a 15 min extension reaction in which DNA polymerase extends an allele-specific primer only if it is perfectly complementary with the target sequence; (iii) detection of the products of the extension reaction within a few minutes by the naked eye employing the dipstick. No purification is required prior to application of the extension products to the dipstick. The method is general and requires only a unique DNA sequence for species discrimination. The only instrument needed is a conventional thermocycler for PCR, which is common equipment in every DNA laboratory. As a model, the method was applied to the discrimination of *Coffea robusta* and *arabica* species in coffee authenticity assessment. As low as 5% of Robusta coffee can be detected in the presence of Arabica coffee.

KEYWORDS: *dipstick, food authentication, single-nucleotide polymorphisms, coffee authenticity*

INTRODUCTION

Authentication is one of the most critical issues in food quality control. In recent years there has been a growing concern among consumers over the composition of food. This has led to an increasing interest toward the development of analytical methods for testing the authenticity of food products, especially the expensive ones.[1−3] Coffee is an important food product, worldwide, and represents an export commodity in more than 50 developing countries in Africa, Asia, and Latin America. Commercially, the most important species are *Coffea arabica* and *Coffea robusta*. Arabica provides superior coffee due to the finer flavor and better quality. Arabica coffee is sold at ∼2−3 times the price of Robusta coffee. Therefore, suitable methods are required, for quality and economical reasons, to differentiate these commodities, thereby ensuring coffee authenticity.

Methods for the discrimination of the two coffee species have been based on the differences in the levels and/or profile of volatile compounds,[4] amino acid enantiomers,[5,6] metals,[7] chlorogenic acid, caffeine, trigonelline,[5,8] sterols,[9] fatty acids,[10,11] tocopherols,[12,13] hydroxycinnamic acid,[14] diterpenoids,[15−17] and polysaccharides.[18]

A DNA-based authenticity test for coffee was reported recently.[19] Phylogenetic studies had previously revealed a number of single-nucleotide polymorphisms (SNP) among several *Coffea* species.[20] As a consequence, species discrimination was achieved through a unique SNP in the chloroplastic *trn*L(UAA)−*trn*F(GAA) intraspacer region that leads to different chlorotypes for Arabica and Robusta. The SNP resides in a *Psu*I restriction site, resulting in the site being present in Robusta but not in Arabica. Consequently, the method involves PCR amplification, restriction enzyme digestion, and restriction fragment analysis by lab-on-a-chip capillary electrophoresis.

The goal of the present work is the development of DNA-based food authenticity assays, in which species identification is accomplished by the naked eye without the need of specialized instruments. Strongly colored nanoparticles (gold nanoparticles) are employed as reporters that enable visual detection. Furthermore, detection is performed in a low-cost, disposable, dipstick-type device that incorporates the required reagents in dry form, thereby avoiding multiple pipetting and incubation steps. Due to its simplicity, the method does not require highly qualified personnel. The procedure comprises the following steps: (i) PCR amplification of the DNA segment that flanks the unique SNP (species marker); (ii) a 15 min extension reaction in which DNA polymerase extends an allele-specific primer only if it is perfectly complementary with the target sequence; (iii) detection of products of the extension reaction within a few minutes by the naked eye employing the dipstick. No purification is required prior to application of the extension products to the dipstick.

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MATERIALS AND METHODS

Instrumentation. PCR and primer extension reactions were performed in an MJ Research PTC-0150 thermocycler (Watertown, MA). A digital camera, Kodak DC 120, and Gel Analyzer, software for DNA documentation, were purchased from Kodak (Rochester, NY). The TLC applicator, Linomat 5, and the software WinCats were from Camag (Muttenz, Switzerland).

Materials. Agarose, deoxynucleotide triphosphates (dNTPs), and DNA molecular weight markers ($\phi \times 174$ DNA) were obtained from HT Biotechnology (Cambridge, U.K.). Biotin-21-dUTP was purchased from Clontech Laboratories (Palo Alto, CA). Ethidium bromide was purchased from Research Organics (Cleveland, OH). Phusion high fidelity DNA polymerase was purchased from Finnzymes (Espoo, Finland). The gold nanoparticles (40 nm, 9×10^{10} particles/mL) were obtained from British Biocell (BB International, Cardiff, U.K.). Immunopore FP nitrocellulose membrane was obtained from Whatman (Florham Park, NJ). The wicking pad, glass fiber conjugate pad, and absorbent pad were obtained from Schleicher & Schuell (Dassel, Germany). Streptavidin from *Streptomyces avidinii* and the High Pure PCR product purification kit were purchased from Roche Diagnostics (Mannheim, Germany). The primers and probes used in this study were synthesized by VBC-Genomics (Wien, Austria). The 20-mer 5′-AATCGATCTGGACGGAAAAGC-3′ was used as an upstream primer (Coffea1-F) and the 25-mer 5′-AGCATCCTCATTTTATGAGAAAAGG-3′ was used as a downstream primer (Coffea1-R) for amplification of the chloroplastic *trn*L(UAA)−*trn*F(GAA) intraspacer region. The 50-mer 5′-$(dA)_{30}$TTCTAGTACCTAGATAAAAT-3′ was used as an allele-specific primer (Usnp4arab) for *C. arabica*. The 50-mer 5′-$(dA)_{30}$TTCTAGTACCTAGATAAAAC-5′ was used as an allele-specific primer (Usnp4rob) for *C. robusta*. A 5′-thiol modified oligo(dT) was used for conjugation with gold nanoparticles. A $(dA)_{30}$ oligonucleotide was used for construction of the control zone of the strip. All other common reagents were purchased from Sigma (St. Louis, MO).

Preparation of Dry Reagent Strip. The dry reagent strip (4 mm × 70 mm) consisted of a wicking pad, a glass fiber conjugate pad, a nitrocellulose diagnostic membrane, an absorbent pad, and a plastic adhesive backing. The strip was assembled as described previously.[21−23] Streptavidin and poly(dA) probe solutions were used for the preparation of the test zone and control zone on the diagnostic membrane, respectively. For this purpose, a solution containing 4 g/L streptavidin, 150 mL/L methanol, and 20 g/L sucrose was loaded at a density of 1.6 μg/4 mm membrane using the TLC applicator. A solution containing 4 μM poly(dA)-tailed probe, 500 mL/L methanol, and 20 g/L sucrose was loaded at a density of 2.4 pmol/4 mm membrane. Poly(dT)-functionalized gold nanoparticles were prepared as described previously.[21−23] The nanoparticles were loaded on the conjugate pad at a density of 3.75 fmol/4 mm. The membranes were dried in an oven for 60 min at 80 °C, and the strips were assembled.

Tailing of Probes with dTTP or dATP. The 5′-thiol-modified $(dT)_{30}$ oligonucleotide and the $(dA)_{30}$ oligonucleotide probes were elongated by tailing with dTTP and dATP, respectively, using terminal deoxynucleotidyl transferase (TdT). Both tailing reactions were performed in a total volume of 20 μL, containing 0.2 M potassium cacodylate (pH 7.2), 0.1 mM dithiothreitol, 0.1 mL/L Triton X-100, 1 mM $CoCl_2$, 3.5 mM dTTP (or dATP), 30 units of TdT, and 700 pmol of probe. The mixture was incubated at 37 °C for 60 min, and the reaction was terminated by the addition of 2 μL of 0.5 M EDTA (pH 8.0). The tailed 5′-thiol-modified poly(dT) probe was purified prior to use by size exclusion chromatography on Sephadex G-25 spin-pure columns. The tailed probes were stored at −20 °C.

DNA Extraction. DNA was extracted from pure and mixed Arabica and Robusta green coffee beans as described in a previous work.[19]

Coffee Authenticity Assessment. *Polymerase Chain Reaction.* PCR was performed in a total volume of 25 μL containing 0.5 unit of Phusion high-fidelity DNA polymerase, 1× Phusion HF reaction buffer, 2 mM $MgCl_2$, 250 μM of each dNTP, 0.25 μM of each primer, and 1 μL of DNA (about 50 ng). The parameters were as follows: initial denaturation at 98 °C for 40 s and 35 cycles of 98 °C for 15 s, 56 °C for 30 s, and 72 °C for 90 s, and final incubation at 72 °C for 7 min. The concentration of the PCR product was determined by agarose gel electrophoresis (2% agarose gel) and ethidium bromide staining followed by densitometric analysis of the picture of the gel taken by a digital camera.

Primer Extension Reaction. All primer extension reactions were carried out in a total volume of 20 μL, containing 20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 1 mL/L Triton X-100, 1 mM $MgSO_4$, 0.25 U of Vent (exo-) DNA polymerase, 0.1 pmol of amplified DNA, 1 pmol of the appropriate primer (Arabica or Robusta specific), 2.5 μM each of dATP, dCTP, and dGTP, 1.875 μM dTTP, and 0.625 μM biotin-21-dUTP. For the analysis of the molar admixtures, mixtures of amplified DNA containing 1, 5, 10, 30, and 50% of Robusta in an Arabica background were prepared. The thermocycling conditions for the primer extension reactions were as follows: an initial denaturation step at 95 °C for 5 min followed by three cycles of denaturation at 95 °C for 15 s, primer annealing for 10 s at 65 °C, and primer extension at 72 °C for 15 s. All primer extension reaction products were subjected to a final denaturation step at 95 °C for 5 min and placed immediately on ice.

Dipstick Assay. A 5 μL aliquot of the denatured extension reaction product was applied onto the conjugate pad next to the gold nanoparticles. The wicking pad was then immersed into a microcentrifuge tube containing 200 μL of 10 mM phosphate buffer (pH 7.4), 75 mM NaCl, 40 mL/L glycerol, and 10 g/L SDS. The visual detection of the extension products was complete within 10 min.

RESULTS AND DISCUSSION

Assay Principle. A 251 bp segment of the chloroplastic *trn*L(UAA)−*trn*F(GAA) intraspacer region is amplified by PCR. This region exhibits three single-nucleotide polymorphisms that lead to different chlorotypes for *C. arabica*

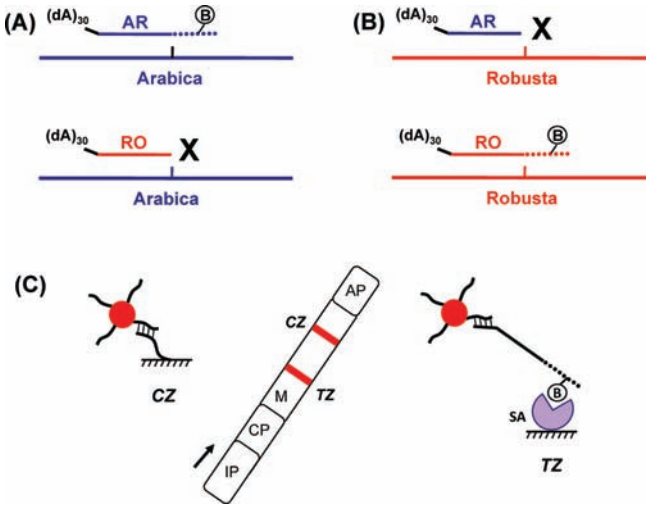

Figure 1. (A) Primer extension reactions of Arabica amplified DNA using the Arabica-specific (AR) and the Robusta-specific primers. (B) Primer extension reactions of Robusta amplified DNA using the Arabica-specific (AR) and the Robusta-specific primers. (C) Dipstick assay configuration of the lateral flow hybridization tests for naked-eye detection of primer extension products. The arrow shows the direction of the flow. IP, immersion pad; CP, conjugate pad; M, membrane; AP, absorbing pad; TZ, test zone; CZ, control zone; SA, streptavidin; B, biotin.

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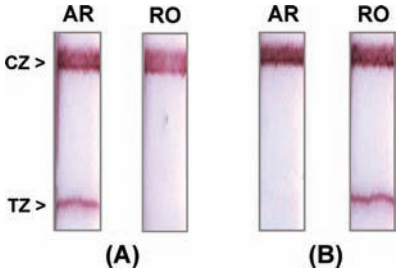

Figure 2. (A) Authenticity test results obtained from a pure Arabica sample. Only the Arabica-specific primer is extended and gives a red line at the test zone of the dipstick. (B) Authenticity test results obtained from a pure Robusta sample. Only the Robusta-specific primer is extended and gives a red line at the test zone of the dipstick. TZ, test zone; CZ, control zone.

and *C. robusta*. One of these SNPs was analyzed according to the proposed method. Following purification, each PCR product was subjected to two separate primer extension reactions using allele-specific primers (Figure 1). Each primer comprises (i) a sequence adjacent to the polymorphic site, with the 3′-end nucleotide complementary to the allelic variant, and (ii) a poly(dA) tag at the 5′-end, for hybridization with the poly(dT) conjugated gold nanoparticles. Due to the high accuracy of nucleotide incorporation by the DNA polymerase, extension occurs only if the primer matches perfectly the target sequence. Biotin-dUTP is incorporated into the extended primer. The extension reaction product is denatured and applied to the conjugate pad of the strip (Figure 1). The bottom part of the strip is immersed into the hybridization buffer. The buffer migrates upward, allowing poly(dA)/(dT) hybridization to occur and linking the extended primer to the nanoparticles. The hybrids migrate along the strip to the test zone (TZ) that contains immobilized streptavidin. If the primer has been extended, the hybrids are captured on the test zone through biotin−streptavidin interaction, forming a red line of accumulated gold nanoparticles. The red color is due to the plasmon resonance peak of the gold nanoparticles at 520 nm. The incorporation of biotin-dUTP is essential for the capture of the hybrids to the test zone. If primer extension has not taken place, then the dA/dT hybridization occurs, but the hybrids are not captured by streptavidin and no red line is observed. The excess nanoparticles are captured by immobilized poly(dA) strands at the control zone (CZ) of the strip, thus forming a red band, which confirms the proper performance of the strip. Dipstick test results obtained from a pure Arabica and a pure Robusta sample are presented in Figure 2.

Optimization of Primer Extension Reaction. Optimization studies of the primer extension reaction for the SNP under investigation were carried out. The goal of optimization was to define the conditions that permit high intensities of the test zones (higher yield of extension products) and specificity in the extension of only the perfectly complementary primers.

First, the ability of the proposed method to discriminate the two species was investigated as a function of the amount of target DNA and the primer/target DNA molar ratio in the primer extension reaction. A series of extension reactions for *C. arabica* DNA fragments were carried out using a constant amount of primer (1 pmol) and various amounts of target DNA in the range of 0−200 fmol. The results are presented in Figure 3. The intensity of the test zone increases with the amount of target used in the extension reaction. Nonspecific signals were practically not observed for all of the target amounts. Thus, 100 fmol of target DNA was chosen as optimum for the discrimination of the two species.

The effect of annealing temperature on the specificity and yield of the extension reaction was investigated in the range of 55−65 °C. The results of this study are also shown in Figure 3. Specificity is not affected by increasing the annealing temperature. The annealing temperature chosen was 65 °C.

Analysis of Mixtures of Amplified DNA from Arabica and Robusta Species. Amplification products generated from pure Arabica and Robusta DNA were mixed at various ratios giving Robusta contents ranging from 1 to 50%. Two separate

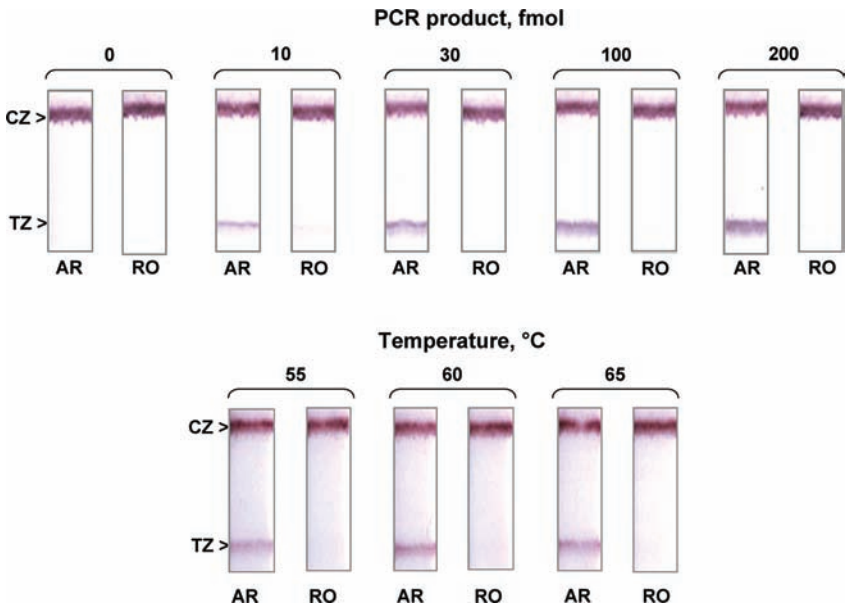

Figure 3. (Upper panel) Effect of the amount of PCR product on the intensity of the test zone of the dipstick. (Lower panel) Effect of the temperature of the primer extension reaction on the intensity of the test zone of the dipstick. AR and RO denote the extension reactions performed by using the Arabica-specific and Robusta-specific primer, respectively. TZ, test zone; CZ, control zone.

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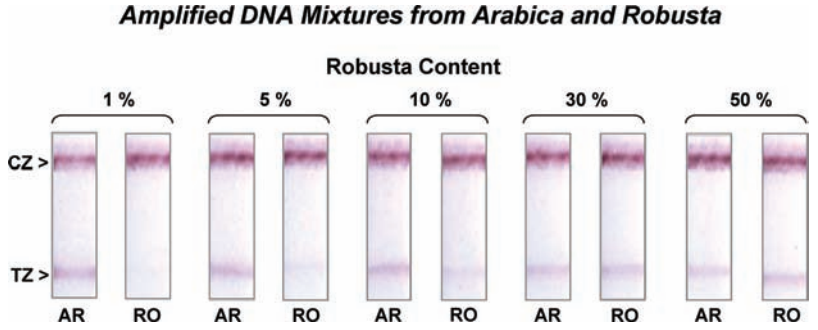

Figure 4. Dipstick test results obtained from mixtures of Arabica and Robusta amplified DNA. The content (percent) of Robusta DNA in the mixture is indicated above the strips. AR and RO denote the primer extension reactions performed by using the Arabica-specific and Robusta-specific primer, respectively. TZ, test zone; CZ, control zone.

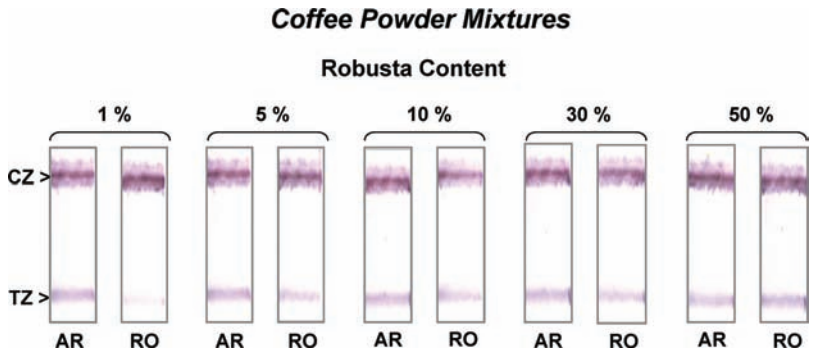

Figure 5. Dipstick test results obtained from mixtures of Arabica and Robusta coffee powders. The content (percent) of Robusta coffee in the mixture is indicated above the strips. AR and RO denote the primer extension reactions performed by using the Arabica-specific and Robusta-specific primer, respectively. TZ, test zone; CZ, control zone.

extension reactions were performed for each mixture using allele-specific primers. The extension products were then analyzed according to the proposed dipstick assay Figure 4. Our results indicate that the proposed method can detect as low as 5% of Robusta DNA in the presence of Arabica DNA and therefore suggest that this approach could be used to authenticate coffee mixtures.

Analysis of Coffee Powder Mixtures. Samples of pure Arabica and Robusta green coffee beans were used to generate a powder. Admixtures of Arabica and Robusta powders were then prepared to give Robusta contents ranging from 1 to 50%. Subsequently, DNA was extracted from these admixtures and amplified by PCR. Following an extension reaction, the products were analyzed according to the proposed dipstick assay. The results are presented in Figure 5. As above, we observe that the method detects as low as 5% of Robusta in the presence of Arabica coffee.

Reproducibility. The reproducibility of the detection of Arabica and Robusta coffees by the dipstick assay was investigated for mixtures containing (A) 10% and (B) 50% of Robusta in the presence of Arabica coffee (n = 3). The density of the test zone was determined by scanning the strip with a desktop scanner (Hewlett-Packard ScanJet 4300C). It was found that the coefficient of variation of the densitometric signal obtained from the test zone ranged from 8.5 to 16.3% for mixture A and from 0.25 to 10.6% for mixture B.

Conclusions. Contrary to a plethora of authentication methods that require specialized and costly instrumentation, the present work introduces a dipstick test that enables DNA-based authenticity testing by the naked eye. As a model, the method was applied to the authentication of coffee. The chloroplastic target using the *trn*L(UAA)−*trn*F(GAA) intergenic spacer region was found to be discriminatory for the Arabica and Robusta varieties. Besides visual detection, the dipstick assay offers the following additional advantages: (a) It allows confirmation of the target DNA sequence by hybridization to a specific probe, contrary to electrophoretic techniques that give only the size of the amplified fragment. (b) It can be used for the authentication of coffee mixtures containing as low as 5% of Robusta. (c) The proposed assay is complete within 25 min after PCR amplification.

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